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(54) Title: TREATMENT FOR CD5⁺ B CELL LYMPHOMA

(57) Abstract: The present invention provides methods for increasing expression of cell surface molecules of CD5⁺ B cell lymphoma cells by contacting cells with immune response modifiers. The invention also provides methods for the treatment of CD5⁺ B cell lymphomas, including chronic lymphocytic leukemia and small lymphocytic lymphoma, by administering immune response modifier compounds to a subject in need of such treatment. Suitable immune response modifier compounds include agonists of TLR7 and/or TLR8.

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TREATMENT FOR CD5⁺ B CELL LYMPHOMA

Background

The peripheral B cell neoplasm chronic lymphoid leukemia/small lymphocytic lymphoma represents the most common lymphoid leukemia. As the name implies, presentation can be as either leukemia or lymphoma. However, the two presentations of this neoplasm, chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL), are morphologically, phenotypically, and genotypically indistinguishable, differing only in the degree of peripheral blood lymphocytosis.

CLL is the most common leukemia of adults in the Western world. In CLL, the peripheral blood contains small, round lymphocytes with scant cytoplasm. Involvement of the bone marrow is observed in all cases of CLL and most cases of SLL, taking the form of interstitial infiltrates or nonparatubular aggregates of small lymphocytes. The tumor cells in CLL and SLL express the pan B cell markers CD29 and CD20. In addition, CD5 - a T cell marker that is expressed only on a small subset of normal B cells - is present on the tumor cells. The immunophenotype of CLL cells is unique. CLL cells co-express the B lymphocyte lineage marker CD19 and the T lymphocyte marker CD5. CLL cells also exhibit a characteristic level of expression of immunoglobulin receptor. Tumor cells typically also have low-level surface expression of Ig heavy chain, with either kappa or lambda light chains.

CLL is a clonal malignancy of B lymphocytes. The disease is usually indolent, with slowly progressive accumulation of long-lived small lymphocytes that are immunoincompetent and respond poorly to antigenic stimulation. CLL is incurable with conventional cytotoxic chemotherapy (Cheson et al., *Blood* 1996; 87:4990-4997; and Keating et al., *Blood* 1993; 81:2878-2884). The hallmark of CLL is isolated lymphocytosis. The white blood cell count is usually greater than 20,000/ μ L and may be markedly elevated to several hundred thousand. The diagnostic requirement for CLL is an absolute lymphocyte count of greater than 4000/ mm^3 . CLL is manifested clinically by immunosuppression, bone marrow failure, and organ infiltration with lymphocytes. Immunodeficiency is also related to inadequate antibody production by the abnormal B cells. With advanced disease, CLL may cause damage by direct tissue infiltration.

In some cases, patients may develop cutaneous lymphoma deposits – erythematous lesions on the skin. The lesions may contain an atypical lymphoid dermal infiltrate of small, round B cells.

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Summary

It has been found that certain small molecule immune response modifiers (IRMs) can be useful for increasing the expression of molecules on the surface of CD5⁺ B cell lymphoma cells. Thus, certain IRMs can be used for treating a CD5⁺ B cell lymphomas such as, for example, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma, or splenic lymphoma with villous lymphocytes.

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Accordingly, the invention provides a method of treating a CD5⁺ B cell lymphoma. Generally, the method includes administering to a subject an IRM compound in an amount effective to ameliorate at least one symptom or clinical sign of the CD5⁺ B cell lymphoma. In some embodiments, administering the IRM compound may result in at least a 50% decrease in peripheral blood lymphocytes, lymphadenopathy, or splenomegaly for at least two months. In other embodiments, administering the IRM compound can inhibit or even prevent the development of progressive disease, wherein progressive disease is at least a 50% increase in circulating lymphocytes or a progression to a more aggressive histology. In still other embodiments, administering the IRM compound can resolve nodular, erythematous lesions associated with CD5⁺ B cell lymphoma.

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In another aspect, the invention provides a method of increasing the expression of at least one cell surface molecule of CD5⁺ B cell lymphoma cells. Generally, the method includes contacting the CD5⁺ B cell lymphoma cells with at least one IRM effective for increasing the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells. In some embodiments, the cell surface molecule may be a costimulatory molecule.

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In another aspect, the present invention also provides a method of stimulating CD5⁺ B cell lymphoma cells to produce a cytokine by contacting the CD5⁺ B cell lymphoma cells with an IRM effective for inducing production of a cytokine above a level produced by the CD5⁺ B cell lymphoma cells not contacted by the IRM. In some embodiments, the cytokine may be IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF- α , GM-CSF, or combinations thereof.

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In another aspect, the invention provides a method of increasing proliferation of CD5⁺ B cell lymphoma-specific cytotoxic T cells. Generally, the method includes contacting CD5⁺ B cell lymphoma cells with an IRM effective to increase the expression of at least one costimulatory molecule on the surface of CD5⁺ B cell lymphoma cells, and then contacting CD8⁺ T cells with the CD5⁺ B cell lymphoma cells, thereby activating the CD8⁺ T cells; wherein the activated T cells are CD5⁺ B cell lymphoma-specific cytotoxic T cells and demonstrate increased proliferation compared to T cells contacted with CD5⁺ B cell lymphoma cells that have not been contacted with an IRM.

In some embodiments, the CD8⁺ T cells are CD5⁺ B cell lymphoma cell-specific.
In other embodiments, the CD8⁺ T cells are naive.

In some embodiments, the IRM compound may be administered to a subject diagnosed as having a CD5⁺ B cell lymphoma so that the activated CD5⁺ B cell lymphoma-specific cytotoxic T cells are autologous CD5⁺ B cell lymphoma-specific cytotoxic T cells.

In some embodiments, the CD5⁺ B cell lymphoma cells may be further contacted with one or more additional immunomodulating agents such as, for example, IL-2 and/or a protein kinase C agonist.

In another aspect, the present invention also provides a method of increasing the killing of CD5⁺ B cell lymphoma cells by cytotoxic T cells. Generally, the method includes contacting CD5⁺ B cell lymphoma cells with an IRM effective to increase the expression of at least one costimulatory molecule on the cell surface of the CD5⁺ lymphoma cells, and then contacting CD8⁺ T cells with the CD5⁺ B cell lymphoma cells, thereby activating the CD8⁺ T cells; wherein the activated CD8⁺ T cells are CD5⁺ B cell lymphoma-specific cytotoxic T cells and demonstrate increased killing of CD5⁺ B cell lymphoma cells compared to T cells contacted with CD5⁺ B cell lymphoma cells that have not been contacted with an IRM.

In some embodiments, the CD8⁺ T cells are CD5⁺ B cell lymphoma cell-specific.
In other embodiments, the CD8⁺ T cells are naive.

In some embodiments, the IRM compound may be administered to a subject diagnosed as having a CD5⁺ B cell lymphoma so that the activated CD5⁺ B cell lymphoma-specific cytotoxic T cells are autologous CD5⁺ B cell lymphoma-specific cytotoxic T cells.

In some embodiments, the CD5⁺ B cell lymphoma cells may be further contacted with one or more additional immunomodulating agents such as, for example, IL-2 and/or a protein kinase C agonist.

5 In another aspect, the present invention also provides a method of treating a subject suffering from a CD5⁺ B cell lymphoma including administering to the subject an IRM effective to increase the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells.

In another aspect, the present invention also provides a vaccine that includes isolated CD5⁺ B cell lymphoma cells, or an immunologically active portion thereof, 10 wherein the isolated CD5⁺ B cell lymphoma cells have been contacted with an IRM effective to increase the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells. In certain embodiments, the CD5⁺ B cell lymphoma cells may be further contacted with one or more additional immunomodulatory agents such as, for example, IL-2 and/or a protein kinase C agonist.

15 In another aspect, the present invention also provides a method of preparing a vaccine including contacting isolated CD5⁺ B cell lymphoma cells with an IRM effective to increase the expression of at least one molecule on the surface of the CD5⁺ B cell lymphoma cells. Some embodiments may include further contacting isolated CD5⁺ B cell lymphoma cells with one or more additional immunomodulatory agents such as, for 20 example, IL-2 or a protein kinase C agonist. In other embodiments, the isolated CD5⁺ B cell lymphoma cells may be derived from a subject diagnosed as having CLL or SLL.

In some embodiments, the CD5⁺ B cell lymphoma cells may be chronic lymphocytic leukemia (CLL) cells, small lymphocytic lymphoma cells (SLL), mantle cell lymphoma cells, splenic lymphoma with villous lymphocytes, or combinations thereof

25 In some embodiments, the IRM is a TLR7 agonist. In some embodiments, the IRM is a TLR8 agonist. In still other embodiments, the IRM compound may be an agonist of both TLR7 and TLR8.

In some embodiments, the IRM may be an imidazoquinoline amine, a tetrahydroimidazoquinoline amine, an imidazopyridine amine, a 1,2-bridged 30 imidazoquinoline amine, a 6,7-fused cycloalkylimidazopyridine amine, an imidazonaphthyridine amine, a tetrahydroimidazonaphthyridine amine, an oxazoloquinoline amine, a thiazoloquinoline amine, an oxazolopyridine amine, a

thiazolopyridine amine, an oxazolophthyrine amine, or a thiazolophthyrine amine. In certain embodiments, the IRM is an imidazoquinoline amine such as, for example, 1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine. In alternative embodiments, the IRM is a tetrahydroimidazoquinoline amine such as, for example, 4-amino-2-(ethoxymethyl)- α,α -dimethyl-6,7,8,9-tetrahydro-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol hydrate. In still other embodiments, the IRM is a sulfonamide substituted imidazoquinoline amine such as, for example, N-[4-(4-amino-2-ethyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl]methanesulfonamide.

In some embodiments, at least one cell surface molecule whose expression is increased may be CD20, CD22, or CD23, and the method further includes administering to the subject a therapeutic agent that has, as a target, the cell surface molecule whose expression is increased. In some embodiments, the expression of more than one cell surface molecule may be increased.

In some embodiments, at least one costimulatory molecule whose expression is increased may be CD40, CD54, CD80, CD83, CD86, CD25, or CD38. In some embodiments, the expression of more than one costimulatory molecule may be increased.

In some embodiments, the CD5⁺ B cell lymphoma cells may be contacted with an IRM *in vitro*. In other embodiments, the CD5⁺ B cell lymphoma cells may be contacted with an IRM *in vivo* such as, for example, in an organ, tissue, or blood of a subject.

Various other features and advantages of the present invention should become readily apparent with reference to the following detailed description, examples, claims and appended drawings. In several places throughout the specification, guidance is provided through lists of examples. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

Brief Description of the Drawings

Figures 1*A-B*. Enhancement of costimulatory molecule expression on CLL cells by an IRM compound.

Figures 2*A-C*. Effect of an IRM compound (with or without IL-2) on costimulatory molecule expression by CLL cells.

Figures 3*A-B*. Effect of an IRM compound on the ability of CLL cells to stimulate cytotoxic T cell proliferation.

Figures 4A-C. Effect of an IRM compound, IL-2, and PKC agonists on costimulatory molecule expression by CLL cells.

Figures 5A-B. Induction of T cell cytotoxicity against autologous CLL cells by PKC agonists, IL-2, and IRM.

5 Figures 6A-B. Photographs of a lymphomatous skin deposit before treatment (Fig. 6a) and after treatment (Fig. 6b) with an IRM.

Figure 7. Effect of IRM1 on costimulatory molecule expression by CLL cells.

Figure 8A-B. Effect of IRM3 on expression of CD80 and CD83 by CLL cells.

10 Figure 9. IRM3-Mediated changes in cell surface molecule expression in CLL cells.

Figure 10. IRM3 + IL-2 increases expression of CD20 on CLL cells.

Detailed Description of Illustrative Embodiments of the Invention

15 The present invention provides methods for treating CD5⁺ B cell lymphomas such as, for example, chronic lymphocytic leukemia (CLL). While a number of clinical observations suggest that CD5⁺ B cell lymphoma cells may be subject to T cell mediated immune recognition (see, for example, Ribera et al., *Blood Cells* 1987; 12:471-483; Ziegler-Heitbrock et al., *Blood* 1989; 73:1426-1430; Wierda et al., *Blood* 2000; 96:2917-2924; Gitelson et al., *Clin Cancer Res.* 2003; 99:1656-1665; and Pavletic et al., *Bone* 20 *Marrow Transplant* 2000; 25:717-722), the weak immunogenicity of CD5⁺ B cell lymphoma cells has limited development of immunologically based treatment methods and, therefore, contributes to disease progression.

25 The present invention demonstrates, for the first time, that contacting CD5⁺ B cell lymphoma cells with an immune response modifier (IRM) compound may be useful for treating CD5⁺ B cell lymphomas. IRM compounds appear to act through basic immune system mechanisms known as toll-like receptors (TLRs) to induce selected biosynthesis of certain cytokines, chemokines and costimulatory molecules. Thus, certain IRM compounds can selectively induce certain aspects - and/or inhibit other aspects - of the immune system. In particular, IRM compounds may increase the expression of cell 30 surface molecules of CD5⁺ B cell lymphoma cells, enhance the immunogenicity of CD5⁺ B cell lymphoma cells, and provide a new immunotherapeutic approach for the treatment of CD5⁺ B cell lymphomas. In some cases, the cell surface molecule whose expression is

increased may be a costimulatory molecule. Increasing the cell surface expression of costimulatory molecules may allow the CD5⁺ B cell lymphoma cells to become competent antigen presenting cells (APCs) capable of initiating and/or maintaining tumor-reactive T cell activity.

5 As used herein, the following terms shall have the following meanings:

"Ameliorate" refers to any reduction in the extent, severity, frequency, and/or likelihood of a symptom or clinical sign characteristic of a particular condition.

"CD5⁺ B cell lymphoma cells" refers to neoplastic cells having a unique immunophenotype that includes co-expression of CD19 and CD5. In addition to
10 expressing the B lymphocyte lineage marker CD19, CD5⁺ B cell lymphoma cells also express the T lymphocyte marker CD5, which is typically expressed only on a small subset of normal B cells. CD5⁺ B cell lymphoma cells include, for example, chronic lymphocytic leukemia (CLL) cells, small lymphocytic lymphoma (SLL) cells, mantle cell lymphoma cells, and splenic lymphoma with villous lymphocytes. In some embodiments, the CD5⁺ B cell lymphoma cells are CLL cells or SLL cells. In certain specific
15 embodiments, the CD5⁺ B cell lymphoma cells are CLL cells.

"Cell surface molecule" refers to a molecule that is expressed on the surface of a cell and may be used to determine the cell's lineage or otherwise may be used to distinguish one cell or cell type from another.

20 "Patient" or "Subject" includes, for example, animals such as, but not limited to, humans, non-human primates, rodents, dogs, cats, horses, pigs, sheep, goats, or cows.

"Sign" or "clinical sign" refers to an objective physical finding relating to a particular condition capable of being found by one other than the patient.

"Symptom" refers to any subjective evidence of disease or of a patient's condition.

25 Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Certain IRM compounds such as, for example, agonists of TLR7 and/or TLR8 can increase the expression of a number of cell surface molecules (including, e.g., costimulatory molecules) of CD5⁺ B cell lymphoma cells, which can result in a more
30 potent immune response being raised against the CD5⁺ B cell lymphoma cells. Thus, increasing expression of cell surface molecules of CD5⁺ B cell lymphoma cells may be exploited to provide therapies that can slow or stop progression of the disease. In certain

embodiments, the therapy may reverse the course of the disease, in some cases even to the point of completely resolving – i.e., curing – the disease.

In addition to having therapeutic utility, CD5⁺ B cell lymphoma cells having increased expression of one or more costimulatory molecules and/or other cell surface molecules may have diagnostic or investigative utility.

Two signals are required for the induction of cell proliferation and cytokine production in naive T cells. The first signal is the foreign antigen, which is presented by self-major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC). The antigenic peptide-MHC complex interacts with a T cell receptor (TCR) on the surface of the naive T cell, thereby providing antigen specificity to the immune response. The second signal is a “costimulatory” signal. Costimulatory signals are antigen-independent and are provided to the naive T cell by the APC, through specific receptor-ligand interactions that promote, for example, T cell survival, clonal expansion, cytokine secretion, and effector function.

In the presence of both signals, a productive adaptive immune response may be generated. In the absence of the costimulatory signal, however, lymphocytes may fail to respond effectively to antigen stimulation. Such unresponsiveness of the adaptive immune system can result in immunologic tolerance.

Accordingly, as used herein, “costimulatory molecule” refers to a member of a subset of cell surface molecules whose expression is necessary, in addition to presentation of a MHC I molecule, to generate a productive immune response, but whose expression is not independently sufficient to generate a productive immune response. Examples of costimulatory molecules include, but are not limited to, members of the B7 family (including, for example, B7-1 (CD80), B7-2 (CD86), ICOS-L (B7RP1), and PDL-1), other molecules of the Ig superfamily (including, for example, CD2 and OX2), molecules of the TNF:TNFR subfamily that lack death domains (including, for example, CD40, OX40, CD27, 4-1BB, and CD30), and some integrins (including, for example, VLA-4, ICAM-1, and ICAM-3).

The increased expression of one or more cell surface molecules can be determined using any of many known methods, including any of the methods described herein. For example, such methods include, but are not limited to, flow cytometry,

immunohistochemistry, reverse transcriptase polymerase chain reaction (RT-PCR), including quantitative RT-PCR, and Northern blot analysis.

In some embodiments, CD5⁺ B cell lymphoma cells may be stimulated to increase expression of one or more of CD20, CD22, CD23, CD25, CD38, CD40, CD54, CD80,
5 CD83, or CD86.

In certain embodiments, the CD5⁺ B cell lymphoma cells may be stimulated to increase expression of a cell surface molecule that is a target of a therapeutic agent such as, for example, a monoclonal antibody that specifically binds to the cell surface molecule. In this way, the increase in cell surface molecule expression may be exploited in a
10 treatment that is targeted against the cell surface molecule. For example, rituximab is a monoclonal antibody that targets CD20 and has been shown to be an effective treatment for non-Hodgkin's lymphoma. Rituximab binds to B cells that express CD20, thereby marking the rituximab-labeled cells for elimination by the immune system. Thus, a treatment that includes, for example, (a) increasing expression of CD20 on CD5⁺ B cell
15 lymphoma cells, and then (b) administering rituximab may permit rituximab to bind to CD5⁺ B cell lymphoma cells, thereby marking CD5⁺ B cell lymphoma cells for elimination by the immune system, and thereby rendering rituximab an effective treatment for CD5⁺ B cell lymphomas. Additional cell surface molecules whose expression may be increased in CD5⁺ B cell lymphoma cells, and that may serve as a target for a therapeutic
20 agent include, for example, CD22 and CD23.

In some embodiments, CD5⁺ B cell lymphoma cells may be stimulated to produce a cytokine by contacting the CD5⁺ B cell lymphoma cells with an IRM effective to produce the cytokine in an amount that is greater than that produced by CD5⁺ B cell lymphoma cells not contacted by the IRM. In some embodiments, the IRM compound
25 may be an agonist of one or more TLRs such as, for example, a TLR7 agonist, a TLR8 agonist, or an agonist of both TLR7 and TLR8. The cytokine produced can include, but is not limited to, IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF- α , GM-CSF, and combinations thereof.

In some embodiments, the CD5⁺ B cell lymphoma cells may be contacted with an IRM compound *in vitro*, for example, in cell culture. In alternative embodiments, CD5⁺ B cell lymphoma cells may be contacted with an IRM compound *in vivo* – i.e., the CD5⁺ B cell lymphoma cells and IRM compound may be contacted in an organ, a tissue, or the
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blood. In such cases, the CD5⁺ B cell lymphoma cells may be contacted with an IRM compound in a subject by, for example, administering an IRM compound to a subject diagnosed as having a CD5⁺ B cell lymphoma. Administration of the IRM directly to the subject allows the CD5⁺ B cell lymphoma cells, after being contacted with IRM, to
5 activate autologous T cells – i.e., the subject's own T cells – thereby generating a T cell-dependent immune response against the CD5⁺ B cell lymphoma cells. By exploiting the subject's own T cell population to generate an immunological response to the CD5⁺ B cell lymphoma cells, one may be able to reduce or even eliminate certain risks associated with therapies that involve administering heterologous biological material (e.g., inflammation,
10 rejection, etc.).

The IRM compounds may be administered via any suitable means, including, for example, parenterally, transdermally, intranasally, and orally. Suitable formulations for delivery of IRM compounds are described in detail below.

In some embodiments, an IRM effective to increase the expression of at least one
15 costimulatory molecule on the cell surface of CD5⁺ B cell lymphoma cells can be administered to a subject suffering from a CD5⁺ B cell lymphoma in a clinically effective amount. As used herein a "clinically effective amount" is an amount effective to demonstrate one or more indications of clinical improvement. Such indications of clinical improvement can include any of those measurements applied in medical practice or
20 laboratory research. See, for example, Cheson et al., National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood*. 1996;87:4990-4997. For example, a clinically effective amount may be an amount effective to obtain a partial response (PR). As used herein, a "partial response" is at least about a 50% decrease in peripheral blood lymphocytes,
25 lymphadenopathy, and/or splenomegaly, for at least two months. A clinically effective amount may be an amount effective to obtain a complete response (CR). As used herein, a "complete response" is the absence of detectable leukemia or lymphoma cells. A clinically effective amount may be an amount effective to prevent progressive disease (PD). As used herein, "progressive disease" is at least about a 50% increase in circulating
30 lymphocytes or the progression to a more aggressive histology, as determined by known pathological criteria. A clinically effective amount may be an amount effective to increase the likelihood or extent of long-term survival. Alternatively, a clinically effective amount

may be an amount that reduces or ameliorates at least one symptom or clinical sign associated with a CD5⁺ B cell lymphoma. For example, a clinically effective amount may be an amount sufficient to reduce the severity, extent, or number of cutaneous lymphoma deposits.

5 In some embodiments, the effect of contacting one or more IRMs with CD5⁺ B cell lymphoma cells may be enhanced by further contacting the CD5⁺ B cell lymphoma cells with one or more additional immunomodulatory agents. In such embodiments, the IRM and one or more additional immunomodulatory agents may be considered a combination such as, for example, a therapeutic combination. Components of such a combination may
10 be said to be delivered “in combination” with one another if the components are provided in any manner that permits the biological effect of contacting one component with CD5⁺ B cell lymphoma cells to be sustained at least until another component is contacted with the CD5⁺ B cell lymphoma cells. Thus, components may be delivered in combination with one another even if they are provided in separate formulations, delivered via different
15 routes of administration, and/or administered at different times.

 A suitable immunomodulatory agent may include, for example, interleukin-2 (“IL-2”). IL-2 is a growth factor for antigen-stimulated T lymphocytes and is responsible for T cell clonal expansion after antigen recognition. IL-2 can be obtained from any of many well-known sources. For example, clinical grade IL-2 can be commercially purchased, for
20 example, from Chiron Corporation, San Francisco, CA.

 In some embodiments, the CD5⁺ B cell lymphoma cells may be contacted with IL-2 *in vitro*, for example, in cell culture. In alternative embodiments, CD5⁺ B cell lymphoma cells may be contacted with IL-2 *in vivo* – i.e., the CD5⁺ B cell lymphoma cells and IL-2 may be contacted in an organ, a tissue, or the blood. In such cases, the CD5⁺ B
25 cell lymphoma cells may be contacted with IL-2 in a subject by, for example, administering IL-2 to a subject suffering from a CD5⁺ B cell lymphoma. The IL-2 may be administered via any suitable means, including, for example, parenterally, transdermally, intranasally, and orally. Suitable formulations for delivery of IL-2 are described below.

 The precise amount of IL-2 used in any one embodiment will vary according to
30 factors known in the art, including but not limited to, the physical and chemical nature of the IL-2, the physical and chemical nature of the IRM or IRMs provided in combination with the IL-2, the intended dosing regimen, the state of the subject’s immune system (e.g.,

suppressed, compromised, stimulated), the method of administering the IL-2, whether any additional immunomodulatory agents are being administered in combination with the IL-2, and the species to which the formulation is being administered. Accordingly it is not practical to set forth generally the amount that constitutes an amount of IL-2 effective for all possible applications. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors. For example, IL-2 may be administered to a subject following procedures similar to those outlined by Rosenberg et al. on the administration of IL-2 for the treatment of melanoma and renal cell carcinoma. Rosenberg et al., *JAMA*, 1994;271:907-913.

Another suitable immunomodulatory agent may include, for example, a protein kinase C (PKC) agonist. Examples of PKC agonists include, but are not limited to, phorbol esters (Totterman et al., *Nature*, 1980;288:176-178) and bryostatin-1 (Drexler et al., *Blood*, 1989;74:1747-1757). Physiological ligands of molecules on the surface of lymphoma cells also can serve as PKC agonists by inducing signal transduction through the cell surface molecules, resulting in the activation of members of the PKC family of proteins. For example, antibodies against certain molecules on the surface of lymphoma cells can also serve as PKC agonists. Such antibodies include, for example, antibodies against MHC class I molecules and antibodies to surface Ig.

In some embodiments, the CD5⁺ B cell lymphoma cells may be contacted with a PKC agonist *in vitro*, for example, in cell culture. In alternative embodiments, CD5⁺ B cell lymphoma cells may be contacted with a PKC agonist *in vivo* – i.e., the CD5⁺ B cell lymphoma cells and a PKC agonist may be contacted in an organ, a tissue, or the blood. In such cases, the CD5⁺ B cell lymphoma cells may be contacted with a PKC agonist in a subject by, for example, administering a PKC agonist to a subject suffering from a CD5⁺ B cell lymphoma. The PKC agonist may be administered via any suitable means, including, for example, parenterally, transdermally, intranasally, and orally. Suitable formulations for delivery of a PKC agonist are described below.

The precise amount of a PKC agonist used in any one embodiment will vary according to factors known in the art including but not limited to the physical and chemical nature of the PKC agonist, the physical and chemical nature of the IRM or IRMs provided in combination with the PKC agonist, the intended dosing regimen, the state of the subject's immune system (e.g., suppressed, compromised, stimulated), the method of

administering the PKC agonist, whether any additional immunomodulatory agents are being administered in combination with the PKC agonist, and the species to which the formulation is being administered. Accordingly it is not practical to set forth generally the amount that constitutes an amount of PKC agonist effective for all possible applications. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

In certain embodiments, CD5⁺ B cell lymphoma cells may be contacted with (a) one or more IRMs effective to increase the expression of at least one costimulatory molecule, (b) IL-2, and (c) a PKC agonist. When CD5⁺ B cell lymphoma cells are contacted with the combination of such an IRM, IL-2, and a PKC agonist, each component of the combination may be provided in a single formulation that includes all of the components. Alternatively, the combination may be provided in two or more formulations, each of which may contain a component of the combination alone or together with one or both of the other components. If the combination is provided in a plurality of formulations, the various formulations may be of similar or dissimilar composition. Furthermore, each formulation may be of similar or dissimilar form (e.g., aerosol, gel, cream, solution, etc.) and may be administered via similar or dissimilar delivery routes (e.g., injection, transdermal, intravenous, etc). Also, if the components of the combination are provided in a plurality of formulations, the various components may be contacted with the CD5⁺ B cell lymphoma cells in any order.

In some embodiments, a result of increasing the expression of at least one costimulatory molecule on the cell surface of CD5⁺ B cell lymphoma cells may include increasing proliferation – i.e., expansion – of CD5⁺ B cell lymphoma cell-specific (hereinafter, “lymphoma cell-specific”) cytotoxic T cells (“CTLs”). Proliferation of lymphoma cell-specific CTLs may result from contacting lymphoma cell-specific CD8⁺ T cells with CD5⁺ B cell lymphoma cells having increased surface expression of costimulatory molecules. Expression of costimulatory molecules on the surface of CD5⁺ B cell lymphoma cells may be increased by any suitable method including, for example, one or more of the methods described above.

In some embodiments, the CD8⁺ T cells are CD5⁺ B cell lymphoma cell-specific – i.e., CD8⁺ T cells to which, or descendants of a CD8⁺ T cell to which, a CD5⁺ B cell lymphoma cell-specific antigen has previously been presented. In other embodiments, the

CD8⁺ T cells are naive - i.e., CD8⁺ T cells to which, or descendants of a CD8⁺ T cell to which, no antigen (CD5⁺ B cell lymphoma-specific or otherwise) has been presented previously.

5 Lymphoma cell-specific CTLs activated by contact with CD5⁺ B cell lymphoma cells having increased expression of at least one costimulatory molecule may exhibit, for example, greater proliferation than that demonstrated by lymphoma cell-specific CTLs activated by contact with CD5⁺ B cell lymphoma cells that do not exhibit increased expression of a one or more costimulatory molecules.

10 In other aspects, the present invention also provides vaccines, methods of making vaccines, and methods of treating a subject by administering a vaccine. Such vaccines include isolated CD5⁺ B cell lymphoma cells, or immunologically active portions thereof, in which the isolated CD5⁺ B cell lymphoma cells have been contacted with an IRM effective to increase the expression of at least one costimulatory molecule on the cell surface. Isolated CD5⁺ B cell lymphoma cells also may be contacted with IL-2, a PKC
15 agonist, or a combination of both IL-2 and a PKC agonist. An immunologically active portion of a CD5⁺ B cell lymphoma cell can include, but is not limited to, a cell membrane preparation and/or a protein preparation from the isolated CD5⁺ B cell lymphoma cells. Thus, for example, a membrane preparation may include portions of the cell membrane from CD5⁺ B cell lymphoma cells and, for example, proteins embedded
20 therein. Vaccines may be made following any of the various procedures for the preparation of cell-based immunizations. For example, methods similar to those used for the preparation of cell-based vaccines against melanoma (see, for example, Wu et al., *J Interferon Cytokine Res.* 2001 Dec;21(12):1117-27), renal cancer cells (see, for example, Vieweg et al., *Urol. Clin. North Am.* 2003 Aug;30(3):633-43) or brain tumors (see, for
25 example, Fecci et al., *J. Neurooncol.* 2003 Aug-Sep;64(1-2):161-76) may be used.

IRMs include compounds that possess potent immunomodulating activity including but not limited to antiviral and antitumor activity. Certain IRMs modulate the production and secretion of cytokines. For example, certain IRM compounds induce the production and secretion of cytokines such as, e.g., Type I interferons, TNF- α , IL-1, IL-6,
30 IL-8, IL-10, IL-12, MIP-1, and/or MCP-1. As another example, certain IRM compounds can inhibit production and secretion of certain T_H2 cytokines, such as IL-4 and IL-5.

Additionally, some IRM compounds are said to suppress IL-1 and TNF (U.S. Patent No. 6,518,265).

Certain IRMs are small organic molecules (e.g., molecular weight under about 1000 Daltons, preferably under about 500 Daltons, as opposed to large biological molecules such as proteins, peptides, and the like) such as those disclosed in, for example, U.S. Patent Nos. 4,689,338; 4,929,624; 4,988,815; 5,037,986; 5,175,296; 5,238,944; 5,266,575; 5,268,376; 5,346,905; 5,352,784; 5,367,076; 5,389,640; 5,395,937; 5,446,153; 5,482,936; 5,693,811; 5,741,908; 5,756,747; 5,939,090; 6,039,969; 6,083,505; 6,110,929; 6,194,425; 6,245,776; 6,331,539; 6,376,669; 6,451,810; 6,525,064; 6,541,485; 6,545,016; 6,545,017; 6,558,951; 6,573,273; 6,656,938; 6,660,735; 6,660,747; 6,664,260; 6,664,264; 6,664,265; 6,667,312; 6,670,372; 6,677,347; 6,677,348; 6,677,349; 6,683,088; 6,756,382; European Patent 0 394 026; U.S. Patent Publication Nos. 2002/0016332; 2002/0055517; 2002/0110840; 2003/0133913; 2003/0199538; and 2004/0014779; and International Patent Publication Nos. WO 01/74343; WO 02/46749 WO 02/102377; WO 03/020889; WO 03/043572; WO 03/045391; WO 03/103584; and WO 04/058759.

Additional examples of small molecule IRMs include certain purine derivatives (such as those described in U.S. Patent Nos. 6,376,501, and 6,028,076), certain imidazoquinoline amide derivatives (such as those described in U.S. Patent No. 6,069,149), certain imidazopyridine derivatives (such as those described in U.S. Patent No. 6,518,265), certain benzimidazole derivatives (such as those described in U.S. Patent 6,387,938), certain derivatives of a 4-aminopyrimidine fused to a five membered nitrogen containing heterocyclic ring (such as adenine derivatives described in U. S. Patent Nos. 6,376,501; 6,028,076 and 6,329,381; and in International Patent Publication No. WO 02/08905), and certain 3- β -D-ribofuranosylthiazolo[4,5-d]pyrimidine derivatives (such as those described in U.S. Publication No. 2003/0199461).

Other IRMs include large biological molecules such as oligonucleotide sequences. Some IRM oligonucleotide sequences contain cytosine-guanine dinucleotides (CpG) and are described, for example, in U.S. Patent Nos. 6,194,388; 6,207,646; 6,239,116; 6,339,068; and 6,406,705. Some CpG-containing oligonucleotides can include synthetic immunomodulatory structural motifs such as those described, for example, in U.S. Patent Nos. 6,426,334 and 6,476,000. Other IRM nucleotide sequences lack CpG sequences and are described, for example, in International Patent Publication No. WO 00/75304.

Other IRMs include biological molecules such as aminoalkyl glucosaminide phosphates (AGPs) and are described, for example, in U.S. Patent Nos. 6,113,918; 6,303,347; 6,525,028; and 6,649,172.

5 Any suitable IRM compound may be used to practice the invention. Unless otherwise indicated, reference to a compound can include the compound in any pharmaceutically acceptable form, including any isomer (e.g., diastereomer or enantiomer), salt, solvate, polymorph, and the like. In particular, if a compound is optically active, reference to the compound can include each of the compound's enantiomers as well as racemic mixtures of the enantiomers.

10 In some embodiments, a suitable IRM compound may be, for example, a small molecule IRM compound such as one of those described above. Suitable small molecule IRM compounds include those having a 2-aminopyridine fused to a five membered nitrogen-containing heterocyclic ring such as, for example, imidazoquinoline amines including but not limited to amide substituted imidazoquinoline amines, sulfonamide
15 substituted imidazoquinoline amines, urea substituted imidazoquinoline amines, aryl ether substituted imidazoquinoline amines, heterocyclic ether substituted imidazoquinoline amines, amido ether substituted imidazoquinoline amines, sulfonamido ether substituted imidazoquinoline amines, urea substituted imidazoquinoline ethers, thioether substituted imidazoquinoline amines, and 6-, 7-, 8-, or 9-aryl or heteroaryl substituted
20 imidazoquinoline amines; tetrahydroimidazoquinoline amines including but not limited to amide substituted tetrahydroimidazoquinoline amines, sulfonamide substituted tetrahydroimidazoquinoline amines, urea substituted tetrahydroimidazoquinoline amines, aryl ether substituted tetrahydroimidazoquinoline amines, heterocyclic ether substituted tetrahydroimidazoquinoline amines, amido ether substituted tetrahydroimidazoquinoline
25 amines, sulfonamido ether substituted tetrahydroimidazoquinoline amines, urea substituted tetrahydroimidazoquinoline ethers, and thioether substituted tetrahydroimidazoquinoline amines; imidazopyridine amines including but not limited to amide substituted imidazopyridine amines, sulfonamido substituted imidazopyridine amines, urea substituted imidazopyridine amines, aryl ether substituted imidazopyridine amines, heterocyclic ether
30 substituted imidazopyridine amines, amido ether substituted imidazopyridine amines, sulfonamido ether substituted imidazopyridine amines, urea substituted imidazopyridine ethers, and thioether substituted imidazopyridine amines; 1,2-bridged imidazoquinoline

amines; 6,7-fused cycloalkylimidazopyridine amines; imidazonaphthyridine amines; tetrahydroimidazonaphthyridine amines; oxazoloquinoline amines; thiazoloquinoline amines; oxazolopyridine amines; thiazolopyridine amines; oxazolonaphthyridine amines; thiazolonaphthyridine amines; and 1*H*-imidazo dimers fused to pyridine amines, quinoline amines, tetrahydroquinoline amines, naphthyridine amines, or tetrahydronaphthyridine amines.

In certain embodiments, the IRM compound can be a tetrahydroimidazoquinoline amine such as, for example, 4-amino-2-(ethoxymethyl)- α,α -dimethyl-6,7,8,9-tetrahydro-1*H*-imidazo[4,5-*c*]quinoline-1-ethanol. In alternative embodiments, the IRM compound may be an imidazoquinoline amine. In certain specific embodiments, the IRM may be 1-(2-methylpropyl)-1*H*-imidazo[4,5-*c*]quinolin-4-amine. In other embodiments, the IRM compound may be a sulfonamide substituted imidazoquinoline amine such as, for example, N-[4-(4-amino-2-ethyl-1*H*-imidazo[4,5-*c*]quinolin-1-yl)butyl]methanesulfonamide. Various combinations of IRMs can be used if desired.

The IRM compound – or each component of a combination such as, for example, an IRM and IL-2 and/or a PKC agonist – may be provided in any formulation suitable for administration to a subject. Suitable types of formulations are described, for example, in U.S. Pat. No. 5,736,553; U.S. Pat. No. 5,238,944; U.S. Pat. No. 5,939,090; U.S. Pat. No. 6,365,166; U.S. Pat. No. 6,245,776; U.S. Pat. No. 6,486,186; European Patent No. EP 0 394 026; and U.S. Patent Publication No. 2003/0199538. The compound – whether an IRM compound, IL-2, or a PKC agonist – may be provided in any suitable form including but not limited to a solution, a suspension, an emulsion, or any form of mixture. The compound may be delivered in formulation with any pharmaceutically acceptable excipient, carrier, or vehicle. For example, the compound may be provided in a formulation suitable for topical administration. Suitable types of formulations for topical delivery of, for example, certain IRM compounds are described, e.g., in International Patent Publication No. WO 03/045391. The formulation may be delivered in any conventional dosage form including but not limited to a cream, an ointment, an aerosol formulation, a non-aerosol spray, a gel, a lotion, a tablet, a lozenge, an elixir, and the like. The formulation may further include one or more additives including but not limited to adjuvants, skin penetration enhancers, colorants, fragrances, moisturizers, thickeners, and the like.

The composition of a formulation suitable for practicing the invention will vary according to factors known in the art including but not limited to the physical and chemical nature of the IRM compound, the nature of the carrier, the intended dosing regimen, the state of the subject's immune system (e.g., suppressed, compromised, stimulated), the method of administering the IRM compound, whether the IRM is being administered in combination with one or more additional agents, and the species to which the formulation is being administered. Accordingly, it is not practical to set forth generally the composition of a formulation effective for all possible applications and all possible embodiments of the invention. Those of ordinary skill in the art, however, can readily determine an appropriate formulation with due consideration of such factors.

In some embodiments, the methods of the present invention include administering IRM to a subject in a formulation of, for example, from about 0.0001% to about 10% (unless otherwise indicated, all percentages provided herein are weight/weight with respect to the total formulation) to the subject, although in some embodiments the IRM compound may be administered using a formulation that provides IRM compound in a concentration outside of this range. In certain embodiments, the method includes administering to a subject a formulation that includes from about 0.01% to about 5% IRM compound, for example, a formulation that includes about 5% IRM compound.

An amount of an IRM compound effective for practicing the invention will vary according to factors known in the art including but not limited to the physical and chemical nature of the IRM compound, the nature of the carrier, the intended dosing regimen, the state of the subject's immune system (e.g., suppressed, compromised, stimulated), the method of administering the IRM compound, whether the IRM is being administered in combination with one or more additional agents, and the species to which the formulation is being administered. Accordingly, it is not practical to set forth generally the amount that constitutes an amount of IRM compound effective for all possible applications and all possible embodiments of the invention. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

In some embodiments, the methods of the present invention include administering sufficient IRM compound to provide a dose of, for example, from about 100 ng/kg to about 50 mg/kg to the subject, although in some embodiments the methods may be

performed by administering IRM compound in a dose outside this range. In some of these embodiments, the method includes administering sufficient IRM compound to provide a dose of from about 10 $\mu\text{g/kg}$ to about 5 mg/kg to the subject, for example, a dose of from about 100 $\mu\text{g/kg}$ to about 1 mg/kg .

5 The dosing regimen may depend at least in part on many factors known in the art including but not limited to the physical and chemical nature of the IRM compound, the nature of the carrier, the amount of IRM being administered, the state of the subject's immune system (e.g., suppressed, compromised, stimulated), the method of administering the IRM compound, whether the IRM is being administered in combination with one or
10 more additional agents, and the species to which the formulation is being administered. Accordingly it is not practical to set forth generally the dosing regimen effective for all possible applications and all possible embodiments of the invention. Those of ordinary skill in the art, however, can readily determine an appropriate dosing regimen with due consideration of such factors.

15 In some embodiments of the invention, the IRM compound may be administered, for example, from a single dose to multiple doses per day. In certain embodiments, the IRM compound may be administered from about three times per week to about once per day. In one particular embodiment, the IRM compound is administered once per day.

20 Examples

The following examples have been selected merely to further illustrate features, advantages, and other details of the invention. It is to be expressly understood, however, that while the examples serve this purpose, the particular materials and amounts used as well as other conditions and details are not to be construed in a matter that would unduly limit the scope of this invention.

The Compounds used in the examples are shown in Table 1.

Table 1

Compound	Chemical Name	Reference
IRM1	4-amino-2-(ethoxymethyl)- α,α -dimethyl-6,7,8,9-tetrahydro-1H-imidazo[4,5-c]quinoline-1-ethanol hydrate	U.S. 5,352,784 Example 91
IRM2	1-(2-methylpropyl)-1H-imidazo[4,5-	U.S. 4,689,338

Compound	Chemical Name	Reference
	<i>c</i>]quinolin-4-amine	Example 99
IRM3	N-[4-(4-amino-2-ethyl-1 <i>H</i> -imidazo[4,5- <i>c</i>]quinolin-1-yl)butyl]methanesulfonamide	U.S. 6,677,349 Example 236
Negative Control (Neg.)	4-hydroxy-1-isobutyl-1 <i>H</i> -imidazo[4,5- <i>c</i>]quinoline	Example 71 of U.S. Patent No. 4,698,348

MATERIALS AND METHODS

Blood samples: Heparinized blood (30-40 mL) was collected from consenting CLL patients (diagnosed by a persistent elevation of CD19⁺CD5⁺IgM^{lo} lymphocytes (Rozman and Montserrat, *New Engl. J. Med.* 1995;333:1052-1057)). All patients were untreated at the time of analysis. Protocols were approved by the appropriate Institutional Review Board.

Table 2

<u>Material</u>	<u>Commercial Source</u>
Lipopolysaccharide (LPS)	Sigma Chemical Co., St. Louis, MO
Phorbol dibutyrate (PDB)	Sigma Chemical Co., St. Louis, MO
Clinical grade IL-2	Chiron Corporation, San Francisco, CA
Interferon- α 2b	Schering Canada, Pointe-Claire, Quebec
Dexamethasone	Pharmascience, Inc., Montreal, Quebec
Bryostatin-1	ICN Biomedicals, Inc., Aurora, OH
Poly (I:C)	Amersham Pharmacia Biotech, Inc., Piscataway, NJ
SB203580	Calbiochem (San Diego, CA)

Antibodies : Phycoerythrin- or FITC-labeled CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), CD83, 4-1BB ligand (4-1BBL), CD5, and CD19 antibodies were purchased from BD Pharmingen (San Francisco, CA). Phycoerythrin-labeled ICOS-L and PDL-1 (B7-H1) antibodies were obtained from eBioscience (San Diego, CA).

Materials Preparation and Methods: Stock solutions of PDB (5 mg/mL) were made in DMSO. Stock solutions of SB203580 (25 mg/mL), an inhibitor of the selective stress-activated protein kinase (SAPK) (p38) (Lee et al., *Pharmacol Ther.* 1999;82:389-397) were made in DMSO. IRM1 and Negative Control (Neg.) compound were provided

by 3M Pharmaceuticals (St. Paul, MN). The compounds were dissolved in AIM-V medium (GibcoBRL, Grand Island, NY)(with 33% DMSO) at 1.3 mg/mL and stored in the dark at 4°C. A 5% cream of IRM2, marketed as ALDARA, also was provided by 3M Pharmaceuticals.

5 *Cell purification:* CLL and T cells were isolated from fresh blood by negative selection (RosetteSep, StemCell Technologies, Vancouver, BC) as described by Gitelson et al. (Gitelson et al., *Clin. Cancer Res.* 2003;99:1656-1665).

10 *Activation of CLL cells:* Purified CLL cells (1.5×10^6 cells/mL) were cultured in serum-free AIM-V medium plus 2-mercaptoethanol (2-ME, 5×10^{-5} M) (Sigma Chemical Co.) in 6- or 24-well plates (Becton-Dickinson Labware, Franklin Lake, NJ) for 3-4 days at 37°C in 5% CO₂. CLL cells were activated by adding the Negative Control compound (1 µg/mL), IRM1 (1 µg/mL), IL-2 (5000 U/mL), PDB (100 ng/mL), or bryostatin (20 nmol), as appropriate. The Negative Control compound did not have measurable effects on CLL cells and, consequently, AIM-V medium, alone, was used as a control for the
15 majority of the experiments.

Mixed Lymphocyte Responses (MLRs): T cells were isolated from CLL patients and adjusted to 5×10^5 cells/mL in AIM-V medium. Activated CLL cells were washed at least 4 times to remove residual immunomodulators, irradiated (2500 cGy) and suspended at 5×10^5 cells/mL (or lower concentrations) in AIM-V. Responders and stimulators were
20 then mixed in a 1:1 (vol:vol) ratio and cultured in 96-well round bottom plates (Becton Dickinson Labware, Franklin Lake, NJ) without additional cytokines or serum. Proliferation was measured 4-6 days later using a colorimetric assay (Gitelson et al., *Clin. Cancer Res.* 2003;99:1656-1665; and Ahmed et al., *J. Immunol. Methods.* 1994;170:211-224).

25 *Flow cytometry:* Cell staining was performed as described by Gitelson et al. (Gitelson et al., *Clin. Cancer Res.* 2003;99:1656-1665).

Cytokine measurement: Cytokine levels in culture supernatants (from activated CLL cells after 48 hours) were determined by a multi-analysis fluorescent bead assay system available from Luminex Corp., Austin, TX, under the tradename LUMINEX-100
30 SYTEM. A 5-plex human cytokine kit for IFN-γ, IL-2, IL-4, IL-10 and TNF-α measurement was used, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Individual cytokine concentrations were determined from standard

curves using software available from BioRad, Mississauga, Ontario, under the tradename BIO-PLEX 2.0. The assay was linear between 30 and 1000 pg/mL for each cytokine.

Statistical analysis: The Student t-test was used to determine p-values for differences between sample means. Best-fit lines were determined by least-squares regression.

EXAMPLE 1: *Effect of IRM1 on costimulatory molecule expression by CLL cells.*

CLL cells from the indicated number of patients were cultured in IRM1 (1 µg/mL) for 3-4 days, and then assayed for expression of the costimulatory molecules indicated on the x-axis (at an intensity greater than the first decade of log fluorescence) of Figure 1A. The percentage of cells that expressed each costimulatory molecule and the mean fluorescence intensity (MFI) of expression were measured by flow cytometry. The “fold-increase” was then calculated from the ratio of these measurements to the percentage and MFI of control cells cultured without activating agents. The average and standard error of these relative increases in costimulatory molecule expression are shown in the Figure 1A.

IRM1 has especially strong effects on CD80, CD86, and CD54 expression, with IRM1 increasing the expression of CD54, CD80, and CD86 on CLL cells from all patients studied (n=31). The effect on CD80 was greater than on CD86 (compare Figures 1, 2). IRM1 also increased the expression of CD83, 4-1BBL, and PDL-1, but had little effect on ICOS-L expression (Figure 1A).

CLL cells from the indicated number of patients were cultured in IRM1, LPS (100 µg/mL), poly (I:C) (100 µg/mL) and IFN-γ 2B (500 U/mL) for 48 hours. Relative increases in the expression of CD80 and CD86 were calculated as described for Figure 1A, and are shown in Figure 1B.

CLL cells were not affected in the same way by other TLR agonists. TLR2 and TLR4 are activated by bacterial LPSs while TLR3 is activated by viral double-stranded RNA and poly (I:C) (Gordon, *Cell*. 2002;111:927-930). However LPS or poly (I:C) rarely affected costimulatory molecule expression by CLL cells (Fig. 1B). Although IRM1 is one of a class of IRMs known to stimulate the release of IFN-α from DCs or monocytes (Gibson et al., *Cell Immunol.* 2002;218:74-86), the effects of IRM1 were unlikely to be mediated indirectly by this cytokine since costimulatory molecule expression by CLL cells did not change significantly after direct stimulation with IFN-γ2B (Fig. 1B).

EXAMPLE 2: *Effects of IL-2 and IRM1 on costimulatory molecule expression by CLL cells.*

CLL cells were isolated and cultured alone or with IL-2 (5000 U/mL), IRM1 (1
5 $\mu\text{g/mL}$), or both IL-2 and IRM1 for 3-4 days. The expression of CD80, CD86, CD54, and
CD83 was then determined by flow cytometry. Figure 2A shows a characteristic example.
The numbers in the dot plots in the upper and lower rows are the percentages of CD80⁺
and CD86⁺ CLL cells, respectively. Figure 2B is a graphical representation of the
percentage of CLL cells expressing the different costimulatory molecules (determined by
10 the percentage of cells with staining intensity above the first decade of log fluorescence)
from the number of patients indicated in the graph legend. The average and standard error
for each of these measurements are shown in the graphs. The numbers over the double-
headed arrows represent the p-values for the differences between sample means. Figure
2C is a graphical representation of the mean fluorescence index (MFI) of expression of the
15 different costimulatory molecules determined for CLL cells from the number of patients
indicated in the graph legend. The average and standard error for each of these
measurements are shown in the graphs. Only the MFI of CD54 expression (divided by 10)
is shown since essentially all CLL cells express this molecule. The numbers over the
double-headed arrows represent the p-values for the differences between sample means.

20 IL-2 and IRM1 both increased the percentage of CLL cells that expressed CD80
and CD86, as well as the mean fluorescence intensity (MFI) of expression of these
molecules (Fig. 2). As a single agent, IRM1 appeared to be more potent than IL-2 in this
regard. The effects of IL-2 and IRM1 on costimulatory molecule expression were additive
(Fig. 2A, right dot-plots and Fig. 2B,C), suggesting they were mediated by different
25 mechanisms. The MFI for the costimulatory molecules CD80, CD86, CD54, and CD83
shown in Fig. 2C indicate that IRM1 increased the expression of all four of these
costimulatory molecules on CLL cells. The magnitude of CD80 expression was especially
increased by IRM1 in combination with IL-2.

Expression of 4-1BBL and PDL-1 was increased somewhat by IL-2 and IRM1, but
30 not as much as CD80, CD86, and CD54 (Fig. 1A). ICOS-L was found on many CLL cells
but its expression appeared to be relatively independent of IL-2 and IRM1-mediated
signaling.

EXAMPLE 3: Effect of PKC activation on costimulatory phenotype and T cell stimulatory ability of IL-2- and IRM1-activated CLL cells.

CLL cells were purified from individual patients and cultured alone or with IL-2, IRM1, IL-2 and IRM1, PDB, PDB and IL-2, PDB and IRM1, or PDB, IL-2, and IRM1. After 3-4 days, these cells were harvested, washed extensively, irradiated (2500 cGy) and used to stimulate allogenic or autologous T cells from CLL patients (obtained at the same time as the CLL cells and rested in culture until added to the Mixed Lymphocytic Response (MLR) assay) after 5-6 days of culture, alamar blue was added and proliferation was measured in an optical density colorimetric microplate reader at wavelengths of 540 (reduced state) and 595 (oxidized state). The difference between these readings was used as a measure of the number of viable cells in the culture. Results are shown in Figure 3A. The results from the T cell source that exhibited the greatest stimulation (after subtraction of the proliferation induced by non-activated CLL cell stimulators) from each individual experiment were used to generate the average proliferation and standard error from the number of patients indicated on the x-axis.

Figure 3B is a graphical representation of the correlation of CD83 expression with T cell stimulatory ability. CLL cells were treated with IL-2 and IRM1 for 4-5 days. The percentage of cells expressing CD83 and the MFI of CD83 expression were then determined by flow cytometry. The activated CLL cells were then irradiated and used to stimulate autologous or allogeneic T cells in MLRs. The initial percentages of CD83⁺ CLL cells (left panel) and the MFI of CD83 expression (right panel) from 19 different patients were then correlated with the measured proliferation in the MLRs. The best straight line has intercept 10.692 and slope 0.0598; the associated P-value is 0.0153.

Figure 4A shows CLL cells from a representative patient that were cultured alone (left panels) or with IRM1, IL-2, and PDB (right panels) for 3 days. CD80, CD83, CD54, and CD86 expression were then determined by flow cytometry. The percentages in the dot-plots refer to CD80 (sum of the right and left upper quadrants) (top panels) and to CD86 (sum of the right upper and lower quadrants) (bottom panels). Figure 4B is a summary of the results of flow cytometric evaluation of the percentage of CLL cells expressing CD80, CD83, CD54, and CD86 (and the MFI of expression) after culture alone, with PDB, PDB and IL-2, PDB and IRM1, or PDB, IL-2 and IRM1 from the

indicated number of patients in the graph legend. The average and standard error are shown. Only the MFI of CD54 expression is shown since essentially all CLL cells express this molecule. Figure 4C is a summary of similar flow cytometric evaluation of ICOS-L, 4-1BBL, and PDL-1 expression. The numbers over the double-headed arrows are the p-values for the differences between sample means.

Treatment with phorbol dibutyrate (PDB), alone, caused ~90% of CLL cells to express CD83 (Fig. 4B, clear bars). PDB also increased the number of CD80⁺ and CD86⁺ CLL cells (the latter more than the former), as well as the expression of 4-1BBL and PDL-1 (Fig. 4C, clear bars). CD54 and ICOS-L expression were not affected greatly by PDB (Fig. 4B and Fig. 4C).

Addition of IL-2 during activation of CLL cells with PDB increased mainly the number of CD80⁺ cells and the MFI of CD80 and CD54 expression (Fig. 4B; horizontal bars). A slightly greater percentage of CD80⁺ cells was obtained when CLL cells were activated with both PDB and IRM1 (Fig. 4B; vertical bars). The addition of IL-2 to IRM1 and PDB strongly increased the expression of CD80 (especially compared to CD86 (Fig. 4B)), as well as CD54, and caused essentially all CLL cells to acquire a CD83^{hi}CD80^{hi}CD86^{hi}CD54^{hi} cell surface phenotype (Fig. 4A and Fig. 4B; diagonal bars).

The results shown in Fig. 4 indicated that the combination of PDB and IRM1 caused nearly 100% of CLL cells to acquire CD80, CD86, and CD83 expression. The addition of IL-2 affected mainly the magnitude of CD80 and CD54 expression. PDB, with or without IL-2, and/or IRM1 increased the expression of 4-1BBL and PDL-1 but not to the same extent as CD80, CD86, CD54, and CD83.

This strong expression of costimulatory molecules by CLL cells activated with PDB, IL-2, and IRM1 was reflected in the ability of these cells to stimulate T cell proliferation (Fig. 3A). CLL cells stimulated with PDB (without IL-2) were weak stimulators of T cell proliferation (Fig. 3A).

EXAMPLE 4: *Elimination of CLL cells by autologous T cells in the presence of IRM1.*

CLL cells and T cells were isolated from a CLL patient, suspended at concentrations of 10⁶ cells/mL and mixed in a 1:1 ratio. The cell mixtures were cultured alone, or in the presence of IL-2, IL-2 and IRM1, bryostatin, bryostatin and IL-2, bryostatin and IRM1, or bryostatin, IL-2, and IRM1. In Figure 5A, after 5 days, the

percentages of CD5⁺CD19⁺ tumor cells (indicated by the numbers in the right upper quadrants of the dot-plots) and CD5⁺CD19⁻ T cells were determined by flow cytometry. In Figure 5B, these percentages and the total numbers of viable cells (determined by manual counting in a hemocytometer) were used to calculate the remaining absolute numbers of CLL cells in the cultures.

IRM1, whether alone or in combination with IL-2 and/or bryostatin, induced autologous T cells to kill CLL cells *in vitro*. The combination of IRM1, IL-2, and bryostatin enabled autologous T cells to achieve 100% clearance of CLL cells in 5 days.

10 **EXAMPLE 5: *Clinical Effects of Administering an IRM compound to Lymphomatous Skin Deposits Associated with Chronic Lymphocytic Leukemia***

A 71-year old Caucasian male was diagnosed with Rai Stage 0 CLL on the basis of a persistent elevated count of circulating monoclonal CD19⁺CD5⁺IgM^{lo} lymphocytes, determined by flow cytometry. CD38 was expressed by 45% of circulating CLL cells. The white blood cell counts at the beginning and end of treatment with IRM2 were 36 x 10⁶ cells/mL and 45 x 10⁶ cells/mL, respectively. Other systemic chemotherapy, steroids, or radiation had not been administered previously.

Additionally, the patient reported having recurrent nodular, erythematous lesions on his hands and arms for approximately eight years. The lesions were usually removed by treatment with liquid nitrogen. At the time he was diagnosed with CLL, he had several such lesions over his upper back (Fig. 8A) and arms. One lesion was biopsied and found to contain a diffuse atypical lymphoid dermal infiltrate consisting of many small, round lymphocytes, without epidermotropism. On paraffin immunoperoxidase stains, the lymphoid filtrate had a predominant CD20⁺ phenotype. Molecular analysis on paraffin embedded tissue demonstrated a monoclonal B cell population, consistent with B cell lymphoma.

A 5% cream of IRM2 was applied to the affected area three times per week. After eight weeks, the size of the treated lesion had not changed significantly, although an area of hypopigmentation had formed that was reminiscent of a halo nevus around a regressing melanoma deposit.

Administration of 5% IRM2 cream was increased to once per day. The lesion disappeared after six weeks of treatment (Fig. 6B) and had not recurred by three months

after treatment ceased. Neither untreated lymphomatous lesions nor circulating white blood cell count changed significantly over the course of the treatment.

CLL cells were isolated from fresh blood by negative selection (RosetteSep, StemCell Technologies, Inc., Vancouver, BC) as described in Gitelson *et al.*, *Clin. Can. Res.*, 9:1656-1665 (2003). Purified CLL cells (1.5×10^6 cells/mL) were cultured in serum-free AIM-V medium (GibcoBRL) for three days. IRM1 and Negative Control compound were used at final concentrations of 1 $\mu\text{g/mL}$.

Cells were incubated with pre-optimized volumes of either CD80-PE and CD83-FITC or CD54-PE and CD86-FITC antibodies for 20 minutes, washed, then subjected to flow cytometry analysis. Negative controls were isotype-matched irrelevant antibodies. Staining of nucleated cells was determined by gating on forward- and side-scatter properties. Ten thousand viable counts were analyzed with a FACScan flow cytometer using CELLQUEST software (BD Immunocytometry Systems, San Jose, CA). The flow cytometer was standardized with SpheroParticles (Spherotech, Inc., Chicago, IL). Percentages of CD80⁺, CD86⁺, and CD83⁺ cells were calculated by comparison with isotype control-labeled cells. Results are shown in Fig. 7.

EXAMPLE 6: Dose Response of IRM3 Effects on Costimulatory Marker Expression by CLL cells

CLL cells from eight different patients were purified and cultured with either 0.001 $\mu\text{g/mL}$, 0.01 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, or 1.0 $\mu\text{g/mL}$ of IRM3 for three days. CD80 and CD83 expression was determined by flow cytometry as described above. Increases in CD80 and CD83 expression were computed by subtracting CD80 and CD83 expression, respectively, from control CLL cultures to which no IRM3 was added. Results are presented in Fig. 8.

EXAMPLE 7: IRM3-Mediated Changes in CLL Cell Surface Molecules

CLL cells were collected from patients and cultured for 2-3 days with IRM3 (1 $\mu\text{g/mL}$) and without (control). The percentages of cells that expressed CD80, CD83, CD86, and CD38 were determined by flow cytometry. The MFI of expression of CD54 and CD25 was determined because virtually all CLL cells express these molecules. Changes in MFI of expression or the percentage of cells expressing a particular cell surface molecule were determined by subtracting values obtained from control cultures

from the values obtained from cells cultured with IRM3. Results are presented in Figure 9.

EXAMPLE 8: *IRM3-Mediated Increase in Expression of CD20 by CLL Cells*

5 CLL cells were purified and cultured either without (control) or with IRM3 (1 μ g/mL) and IL-2 (5000 U/mL). After 48 hours, the MFI of CD20 expression was determined by flow cytometry. Results are presented in Fig.10.

10 The complete disclosures of the patents, patent documents and publications cited herein are incorporated by reference in their entirety as if each were individually incorporated. In case of conflict, the present specification, including definitions, shall control. Various modifications and alterations to this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention. Illustrative embodiments and examples are provided as examples only and are not
15 intended to limit the scope of the present invention. The scope of the invention is limited only by the claims set forth as follows.

What is claimed is:

1. A method of increasing the expression of at least one cell surface molecule of CD5⁺ B cell lymphoma cells, the method comprising contacting the CD5⁺ B cell
5 lymphoma cells with at least one IRM compound effective to increase the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells.
2. The method of claim 1 wherein the CD5⁺ B cell lymphoma cells are selected from the group consisting of chronic lymphocytic leukemia (CLL) cells, small lymphocytic
10 lymphoma cells (SLL), mantle cell lymphoma cells, splenic lymphoma with villous lymphocytes, and combinations thereof.
3. The method of claim 1 wherein the IRM is a TLR7 agonist.
- 15 4. The method of claim 1 wherein the IRM is a TLR8 agonist.
5. The method of claim 1 wherein the IRM is a tetrahydroimidazoquinoline amine.
6. The method of claim 1 wherein the IRM is a sulfonamide substituted
20 imidazoquinoline amine.
7. The method of claim 1 wherein the cell surface molecule is a costimulatory molecule.
- 25 8. The method of claim 7 wherein at least one costimulatory molecule is CD25, CD38, CD40, CD54, CD80, CD83, or CD86.
9. The method of claim 1 wherein the cell surface molecule is CD20, CD22, or
30 CD23.
10. The method of claim 1 wherein contacting the CD5⁺ B cell lymphoma cells with an IRM occurs *in vitro*.

11. The method of claim 1 wherein contacting the CD5⁺ B cell lymphoma cells with an IRM occurs in an organ, tissue, or blood.

5 12. The method of claim 1 wherein contacting the CD5⁺ B cell lymphoma cells with an IRM occurs *in vivo* in a subject.

10 13. A method of stimulating CD5⁺ B cell lymphoma cells to produce a cytokine, the method comprising contacting the CD5⁺ B cell lymphoma cells with an IRM effective to produce at least one cytokine above a level produced by the CD5⁺ B cell lymphoma cells not contacted by the IRM, wherein the at least one cytokine is IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF- α , GM-CSF, or a combination thereof.

15 14. The method of claim 13 wherein contacting the CD5⁺ B cell lymphoma cells occurs *in vitro*.

15. The method of claim 13 wherein contacting the CD5⁺ B cell lymphoma cells occurs in an organ, tissue, or blood.

20 16. The method of claim 13 wherein contacting the CD5⁺ B cell lymphoma cells occurs *in vivo* in a subject.

17. A method of increasing T cell proliferation, the method comprising:
contacting CD5⁺ B cell lymphoma cells with an IRM effective to increase the
25 expression of at least one costimulatory molecule on the cell surface of CD5⁺ B cell lymphoma cells; and
contacting the CD5⁺ B cell lymphoma cells with T cells, thereby activating the T cells;
wherein the activated T cells demonstrate increased proliferation compared to T
30 cells contacted with CD5⁺ B cell lymphoma cells that have not been contacted with an IRM.

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18. The method of claim 17, further comprising contacting the CD5⁺ B cell lymphoma cells with at least one additional immunomodulating agent.

19. The method of claim 18 wherein the additional immunomodulating agent
5 comprises IL-2.

20. The method of claim 18 wherein the additional immunomodulating agent comprises a protein kinase C agonist.

10 21. The method of claim 18 wherein the T cells and the CD5⁺ B cell lymphoma cells are contacted *in vivo*.

22. A method of increasing the killing of CD5⁺ B cell lymphoma cells by CD5⁺ B cell lymphoma cell-specific cytotoxic T cells, the method comprising:

15 contacting CD5⁺ B cell lymphoma cells with an IRM effective to increase the expression of at least one costimulatory molecule on the surface of the CD5⁺ lymphoma cells; and

 contacting the CD5⁺ lymphoma cells with CD8⁺ T cells, thereby activating the CD8⁺ T cells;

20 wherein the activated CD8⁺ T cells are CD5⁺ B cell lymphoma cell-specific cytotoxic T cells that demonstrate increased killing of CD5⁺ B cell lymphoma cells compared to CD8⁺ T cells contacted with CD5⁺ B cell lymphoma cells that have not been contacted with an IRM.

25 23. The method of claim 22, further comprising contacting the CD5⁺ B cell lymphoma cells with at least one additional immunomodulating agent.

24. The method of claim 23 wherein the additional immunomodulating agent
30 comprises IL-2.

25. The method of claim 23 wherein the additional immunomodulating agent comprises a protein kinase C agonist.

26. The method of claim 22 wherein the CD5⁺ B cell lymphoma cells are contacted with the IRM *in vivo*.
- 5 27. A method of increasing the killing of CD5⁺ B cell lymphoma cells by autologous T cells in a subject suffering from a CD5⁺ B cell lymphoma, the method comprising administering to the subject an IRM that is a TLR7 and/or TLR8 agonist.
28. The method of claim 27 further comprising administering IL-2.
- 10 29. The method of claim 27 further comprising administering a protein kinase C agonist.
30. The method of claim 27 further comprising administering IL-2 and a protein kinase C agonist.
- 15 31. A method of treating a subject suffering from a CD5⁺ B cell lymphoma, the method comprising administering to the subject an IRM effective to increase the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells in an amount effective to increase the expression of at least one cell surface molecule of the CD5⁺ B cell lymphoma cells.
- 20 32. The method of claim 31 wherein the IRM is a TLR7 and/or TLR8 agonist.
- 25 33. The method of claim 31 further comprising administering IL-2.
34. The method of claim 31 further comprising administering a protein kinase C agonist.
- 30 35. The method of claim 31 further comprising administering IL-2 and a protein kinase C agonist.

36. The method of claim 31 wherein at least one cell surface molecule whose expression is increased is a target of a therapeutic agent, and the method further comprises administering to the subject an effective amount of the therapeutic agent.

5 37. The method of claim 26 wherein the cell surface molecule is CD20, CD22, or CD23.

38. The method of claim 31 wherein the cell surface molecule is a costimulatory molecule.

10

39. A method of treating a CD5⁺ B cell lymphoma, the method comprising administering to a subject in need of such treatment an IRM effective to ameliorate at least one symptom or clinical sign characteristic of a the CD5⁺ B cell lymphoma.

15 40. The method of claim 39 wherein the IRM is administered in an amount effective to demonstrate at least a 50% decrease in peripheral blood lymphocytes, lymphadenopathy, or splenomegaly for at least two months.

20 41. The method of claim 39 wherein the IRM is administered in an amount effective to prevent the development of progressive disease, wherein progressive disease is at least a 50% increase in circulating lymphocytes or a progression to a more aggressive histology.

42. The method of claim 39 wherein the IRM is administered in an amount effective to ameliorate erythematous lesions.

25

43. A vaccine comprising isolated CD5⁺ B cell lymphoma cells or an immunologically active portion thereof, wherein isolated CD5⁺ B cell lymphoma cells have been contacted with an IRM effective to increase the expression of at least one costimulatory molecule on the cell surface of the CD5⁺ B cell lymphoma cells.

30

44. The vaccine of claim 43 wherein the IRM is a TLR7 and/or TLR8 agonist.

45. The vaccine of claim 43 wherein the isolated CD5⁺ B cell lymphoma cells have further been contacted with IL-2.

5 46. The vaccine of claim 43 wherein the isolated CD5⁺ B cell lymphoma cells have further been contacted with a protein kinase C agonist.

47. The vaccine of claim 43 wherein the isolated CD5⁺ B cell lymphoma cells have further been contacted with IL-2 and a protein kinase C agonist.

10 48. A method of preparing a vaccine comprising contacting isolated CD5⁺ B cell lymphoma cells with an IRM effective to increase the expression of at least one molecule on the surface of the CD5⁺ B cell lymphoma cells.

15 49. The method of claim 48 further comprising contacting said isolated cells with IL-2.

50. The method of claim 48 further comprising contacting said isolated cells with a protein kinase C agonist.

20 51. The method of claim 48 further comprising contacting said isolated cells with IL-2 and a protein kinase C agonist.

25 52. A method of treating a subject suffering from a CD5⁺ lymphoma, the method comprising administering to the subject an immunologically active portion of isolated CD5⁺ B cell lymphoma cells, wherein the isolated CD5⁺ B cell lymphoma cells have been contacted with an IRM effective to increase the expression of at least one costimulatory molecule on the cell surface of the CD5⁺ B cell lymphoma cells.

30 53. The method of claim 52 wherein the isolated CD5⁺ B cell lymphoma cells have also been contacted with IL-2.

54. The method of claim 52 wherein the isolated the CD5⁺ B cell lymphoma cells have also been contacted with a protein kinase C agonist.

55. The method of claim 52 wherein the isolated CD5⁺ B cell lymphoma cells have also been contacted with IL-2 and a protein kinase C agonist.

5 56. The method of claim 52 wherein the isolated CD5⁺ B cell lymphoma cells are derived from the subject suffering from a CD5⁺ B cell lymphoma.

57. The method of claim 52 wherein the immunologically active portion of isolated CD5⁺ B cell lymphoma cells comprises whole cells.

10

58. The method of claim 52 wherein the immunologically active portion of isolated CD5⁺ B cell lymphoma cells comprises a cell membrane preparation or a protein preparation from the isolated CD5⁺ B cell lymphoma cells.

15 59. The method of claim 52 wherein at least one cell surface molecule whose expression is increased is a target of a therapeutic agent, and the method further comprises administering to the subject an effective amount of the therapeutic agent.

20 60. A method of initiating a CD5⁺ B cell lymphoma-reactive T cell response in a subject diagnosed as having a CD5⁺ B cell lymphoma, the method comprising administering to the subject a composition comprising an immunologically active portion of isolated CD5⁺ B cell lymphoma cells that have been contacted with an IRM effective to increase the expression of at least one costimulatory molecule on the cell surface of the CD5⁺ B cell lymphoma cells.

25

61. The method of claim 60 wherein the isolated CD5⁺ B cell lymphoma cells have also been contacted with IL-2.

30 62. The method of claim 60 wherein the isolated CD5⁺ B cell lymphoma cells have also been contacted with a protein kinase C agonist.

63. The method of claim 60 wherein the isolated CD5⁺ B cell lymphoma cells have also been contacted with IL-2 and a protein kinase C agonist.
- 5 64. The method of claim 60 wherein the isolated CD5⁺ B cell lymphoma cells are derived from the subject suffering from the CD5⁺ lymphoma.
65. The method of claim 60 wherein the isolated CD5⁺ B cell lymphoma cells are derived from a subject suffering from CLL or SLL.
- 10 66. The method of claim 60 wherein the immunologically active portion of isolated CD5⁺ B cell lymphoma cells comprises whole cells.
- 15 67. The method of claim 60 wherein the immunologically active portion of isolated CD5⁺ B cell lymphoma cells comprises a cell membrane preparation or a protein preparation from the isolated CD5⁺ B cell lymphoma cells.

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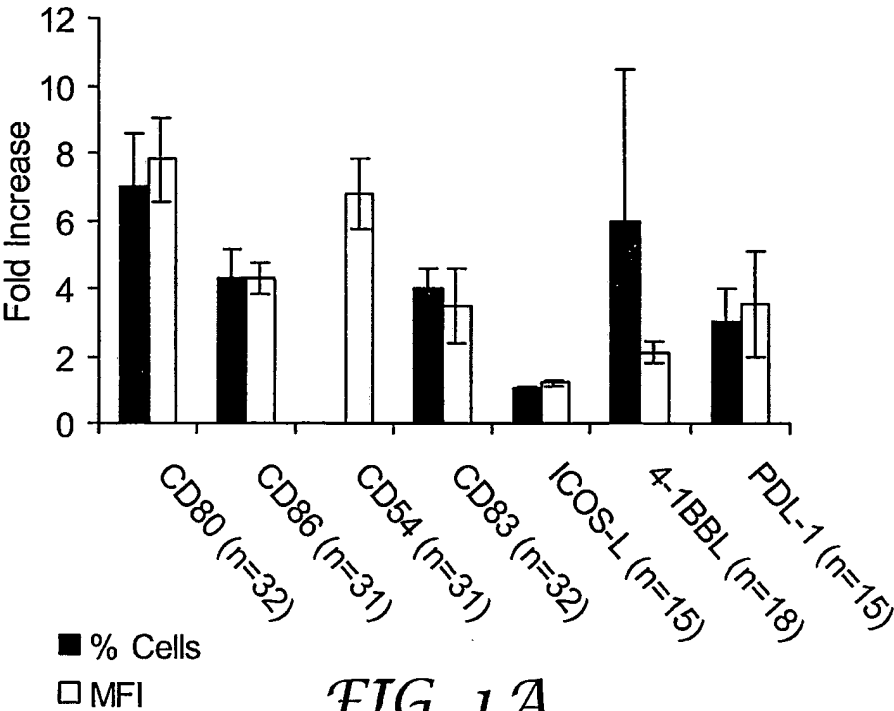


FIG. 1A

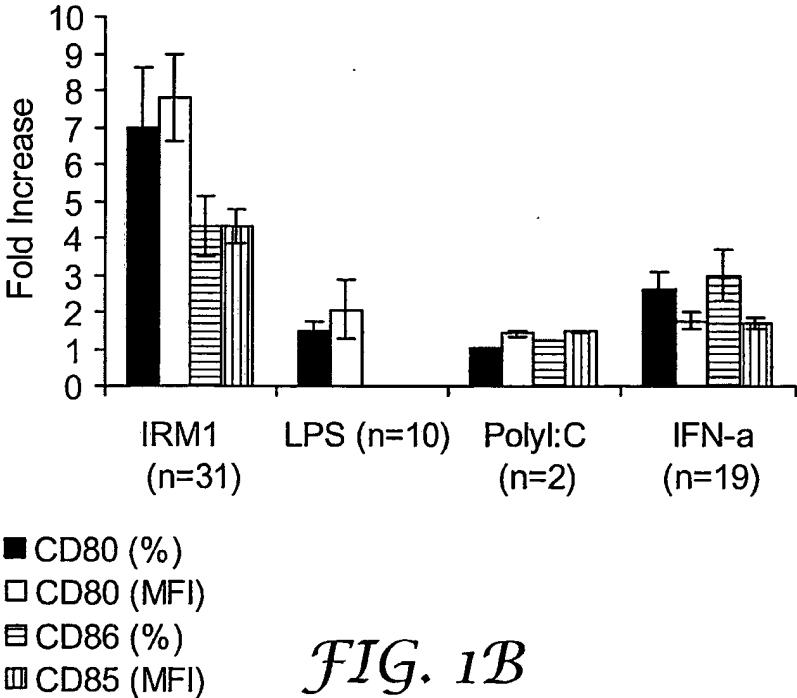
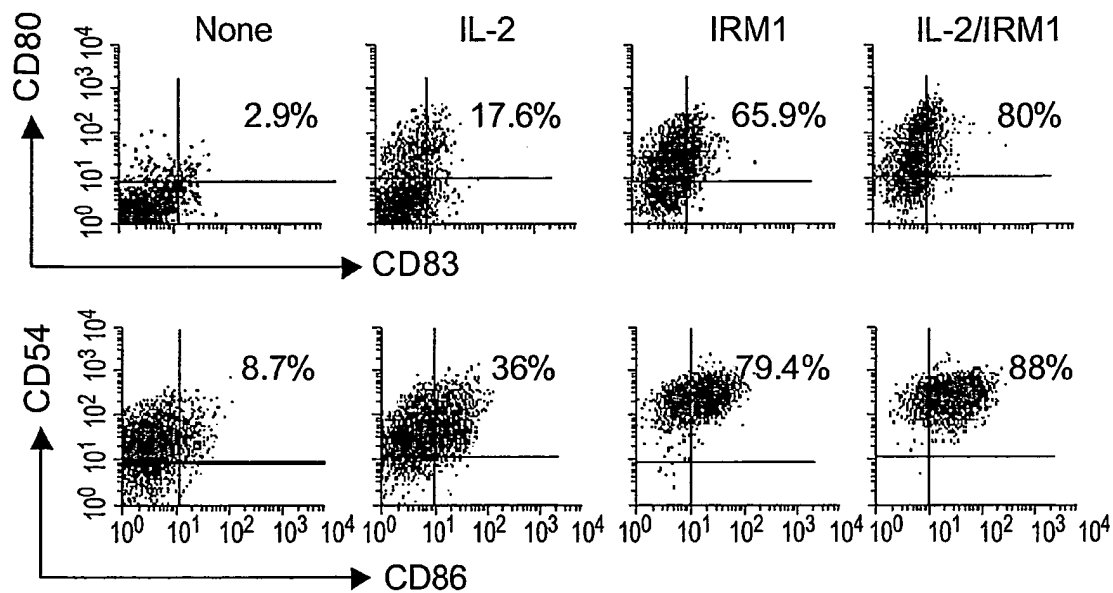
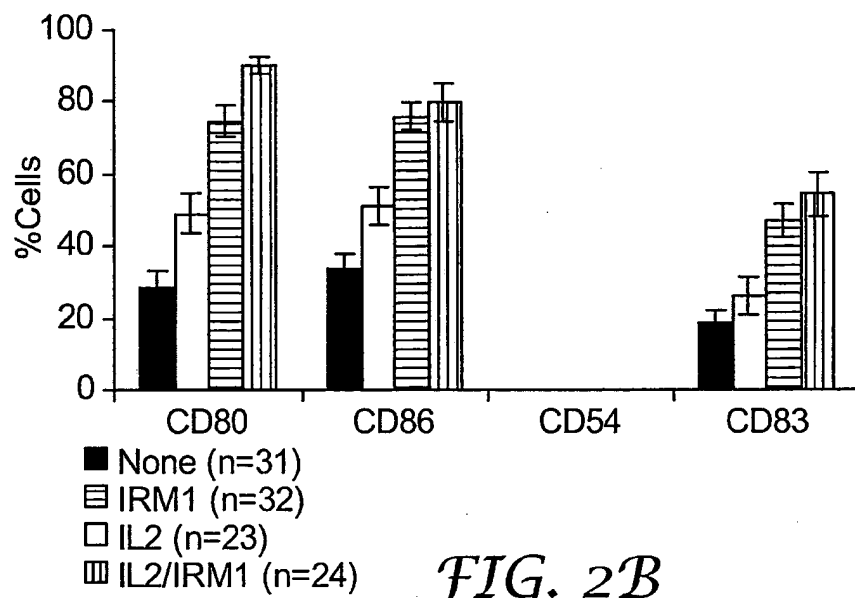
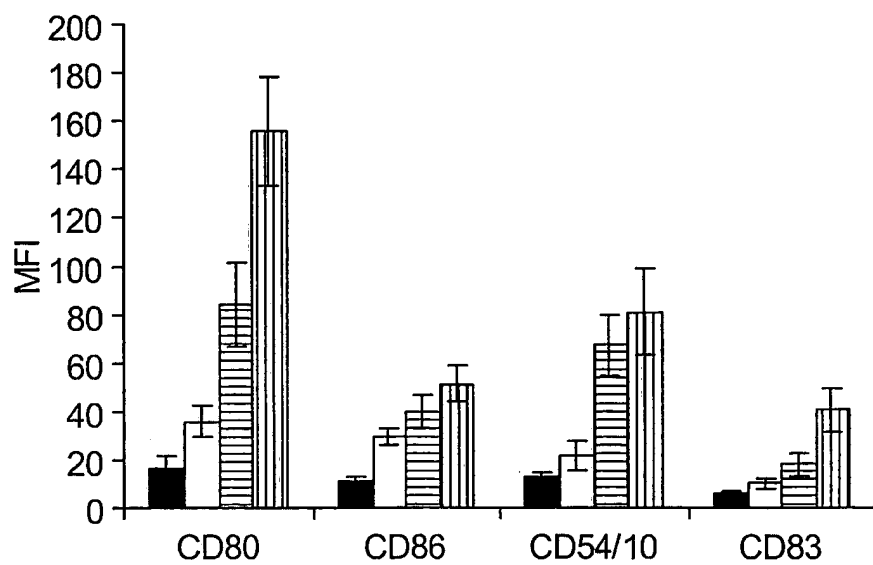


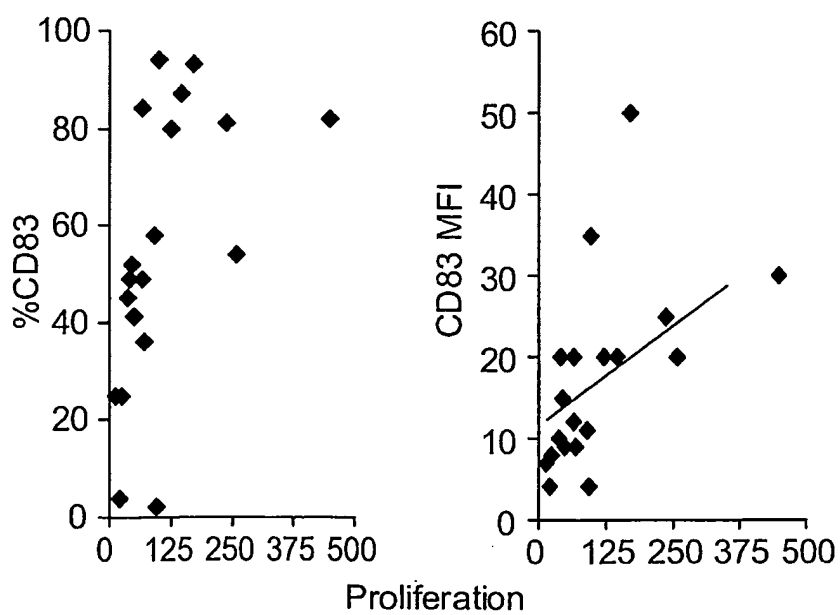
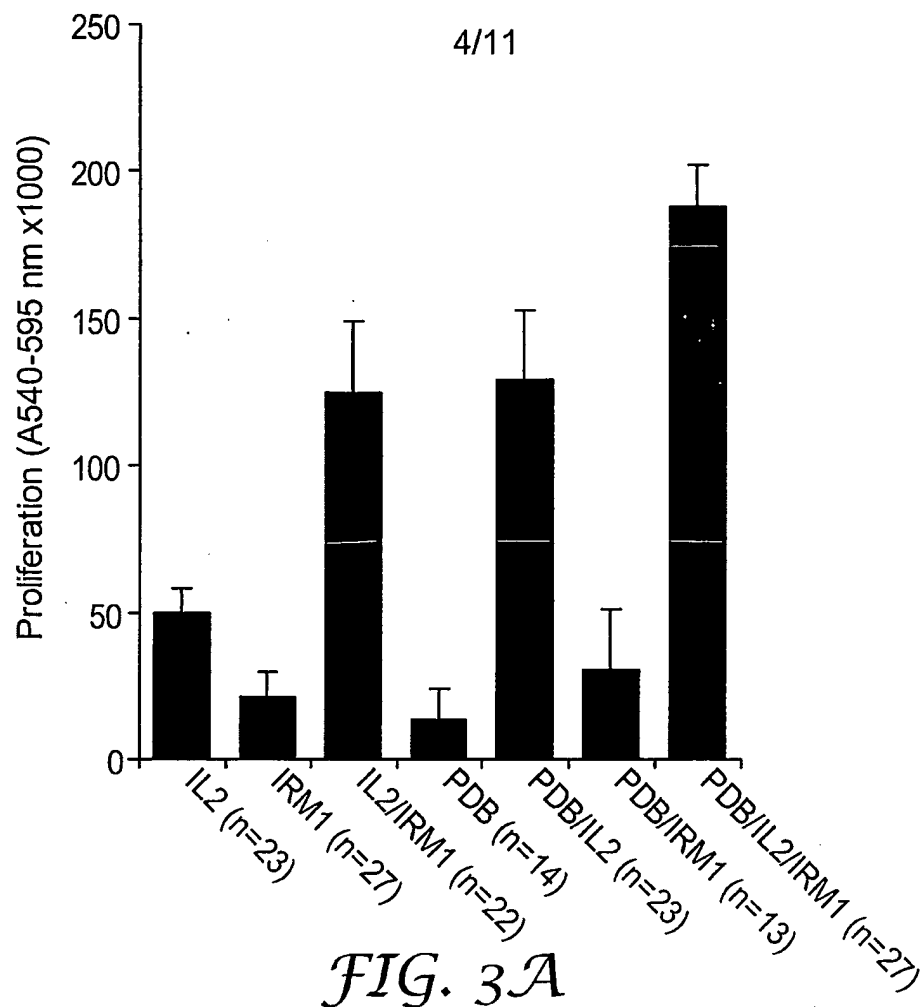
FIG. 1B

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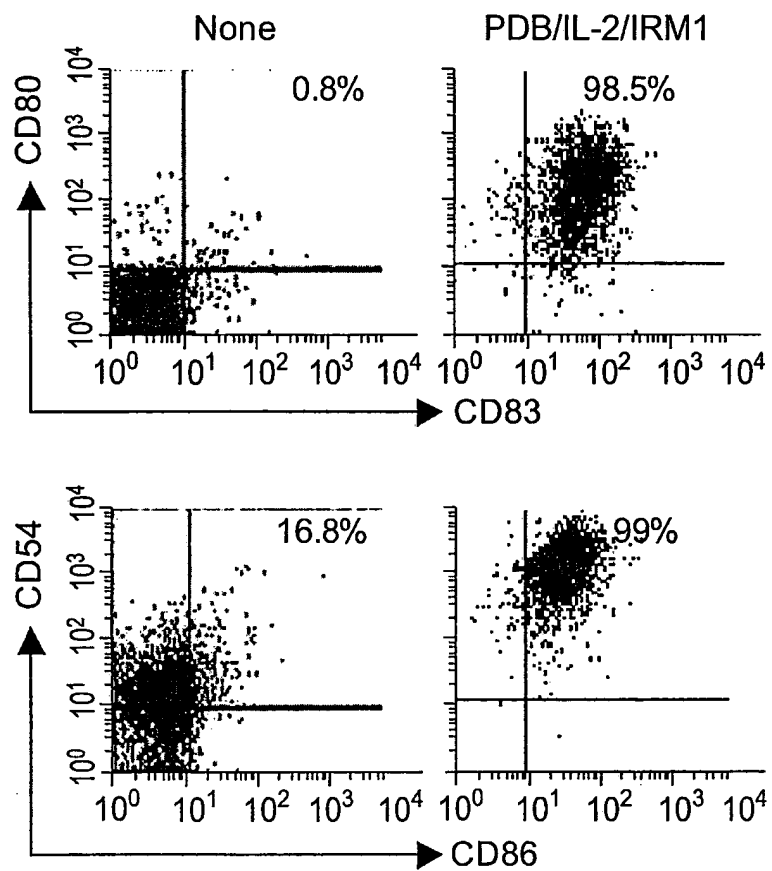
*FIG. 2A*

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*FIG. 2B**FIG. 2C*



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*FIG. 4A*

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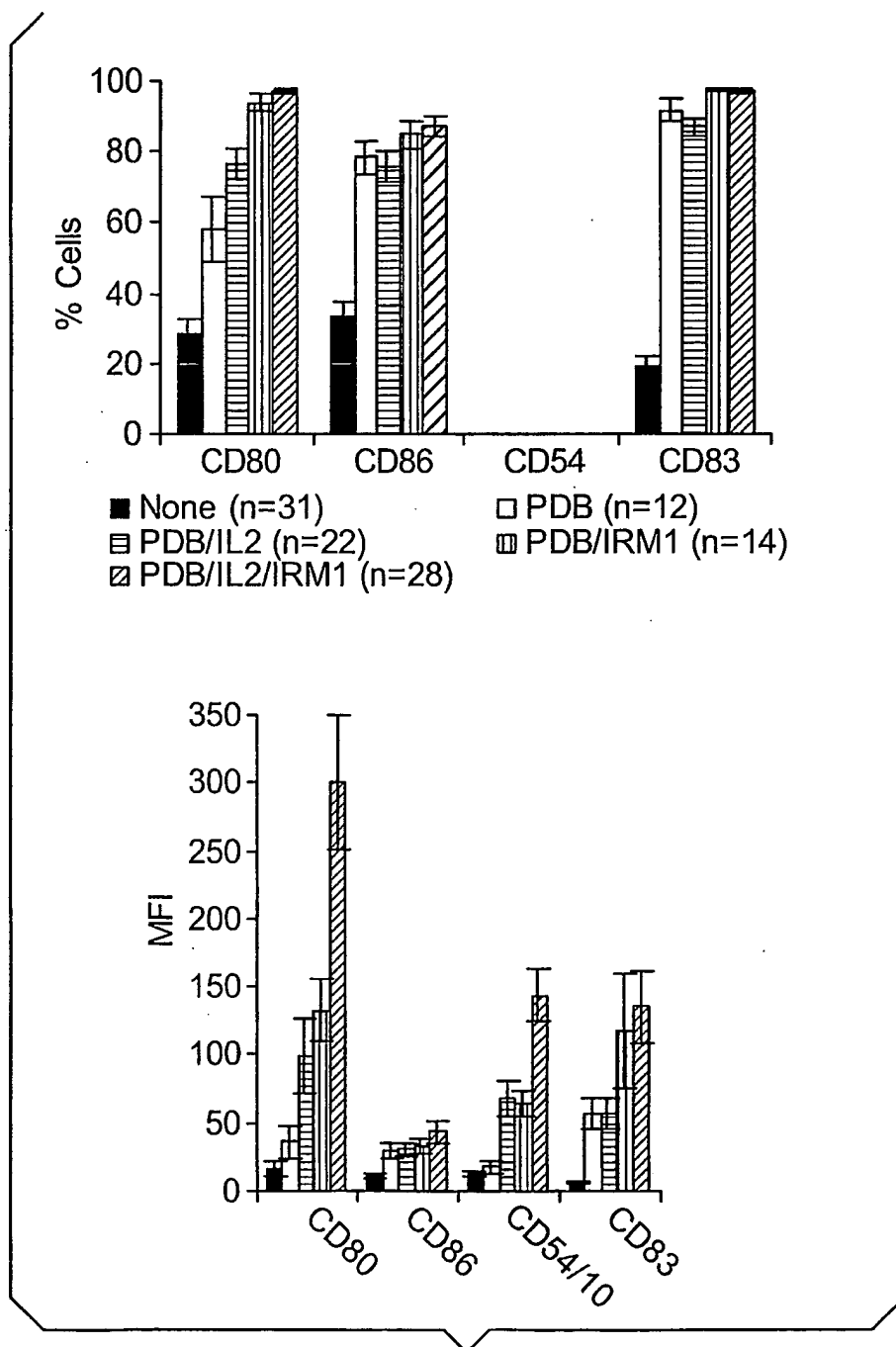


FIG. 4B

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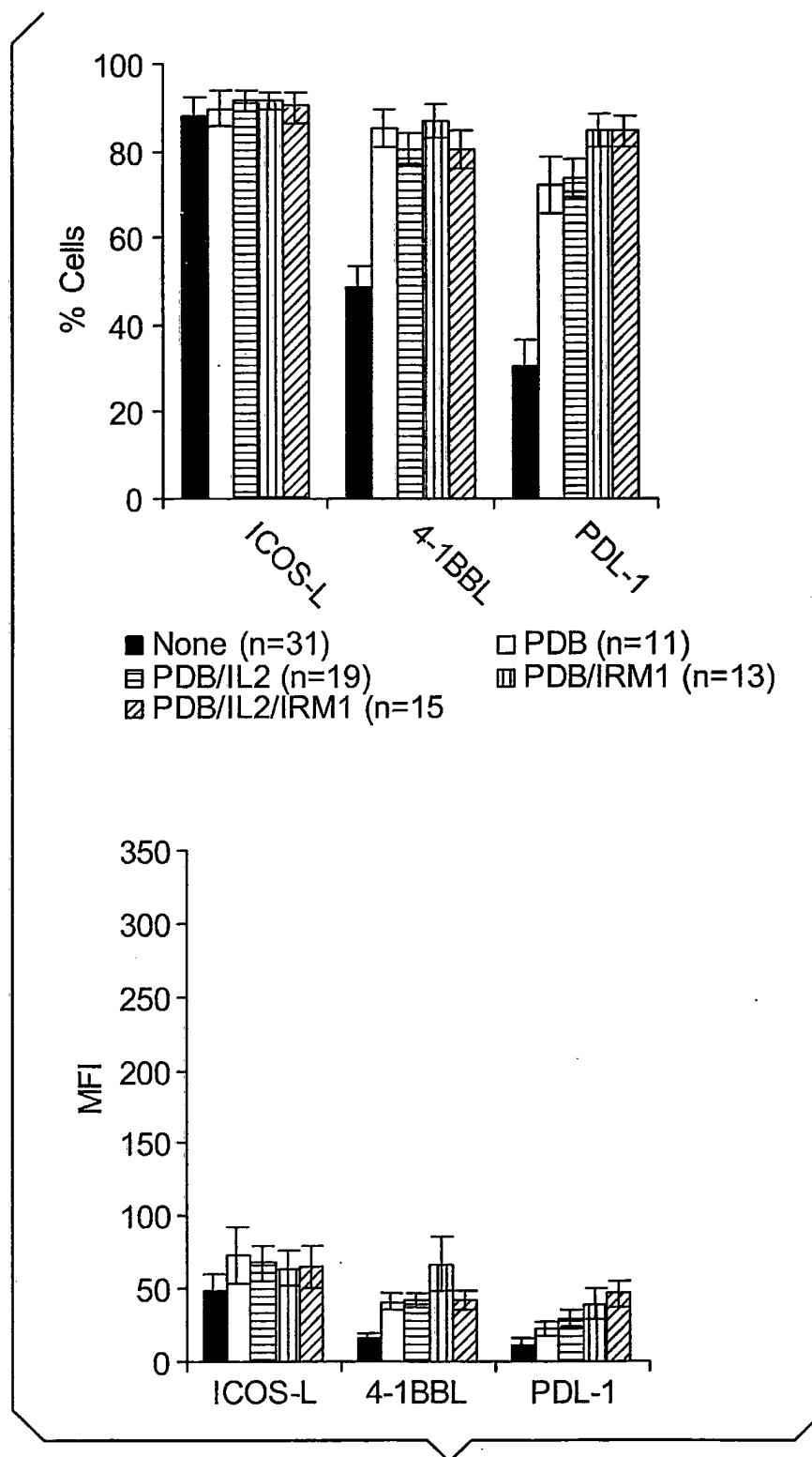
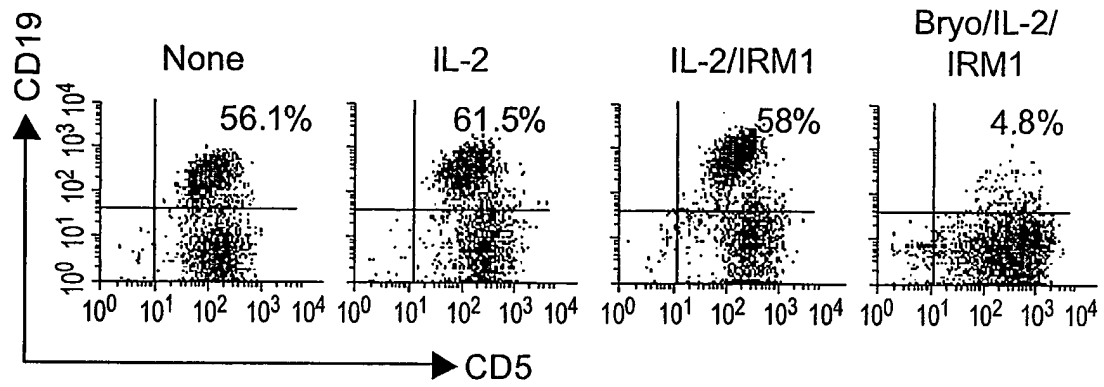
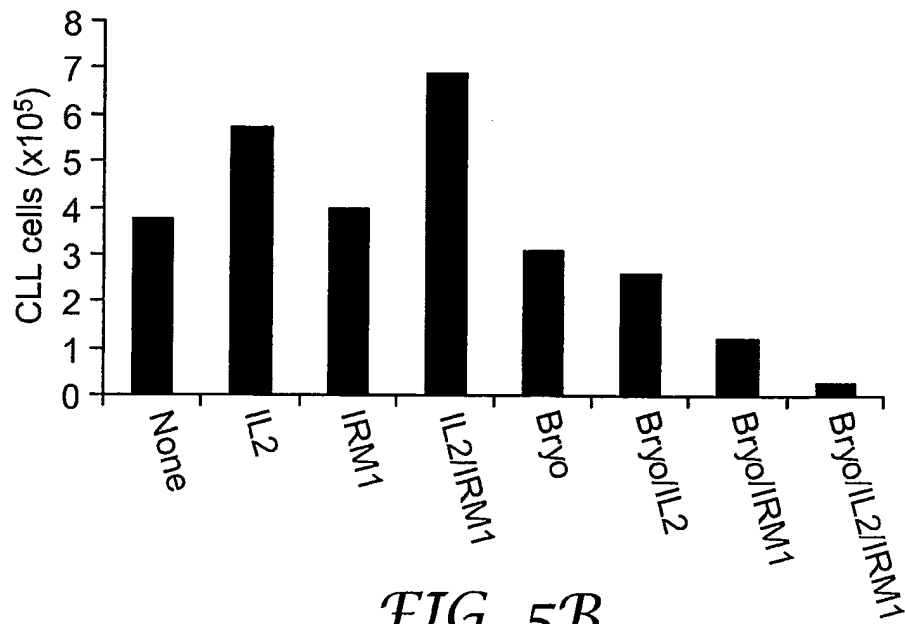
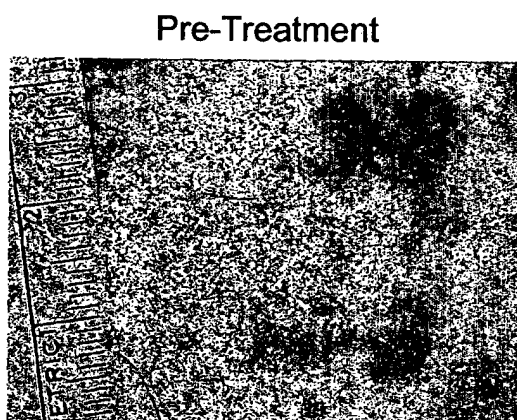
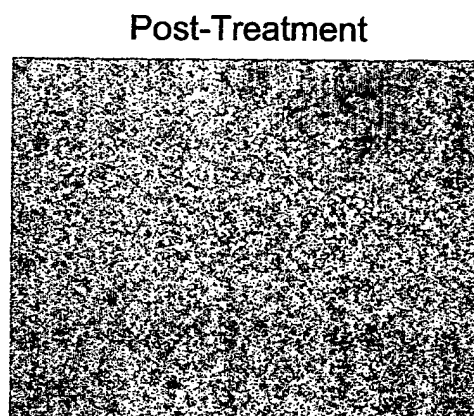
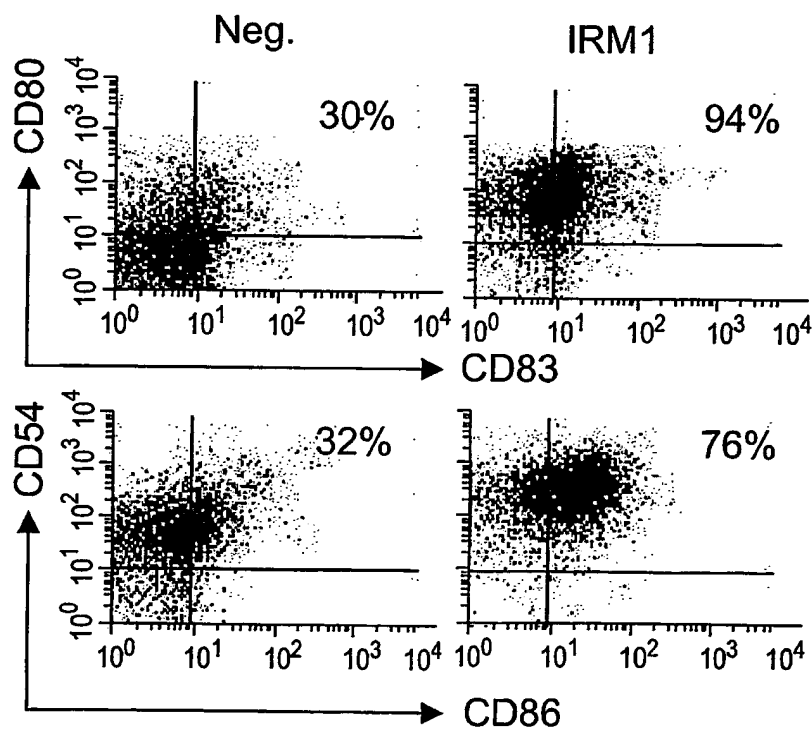


FIG. 4C

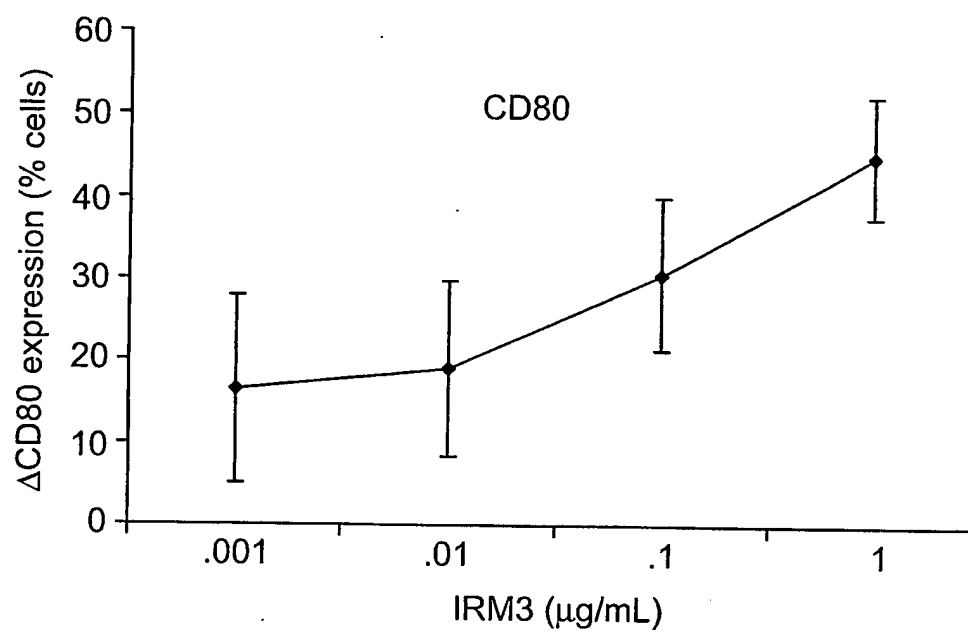
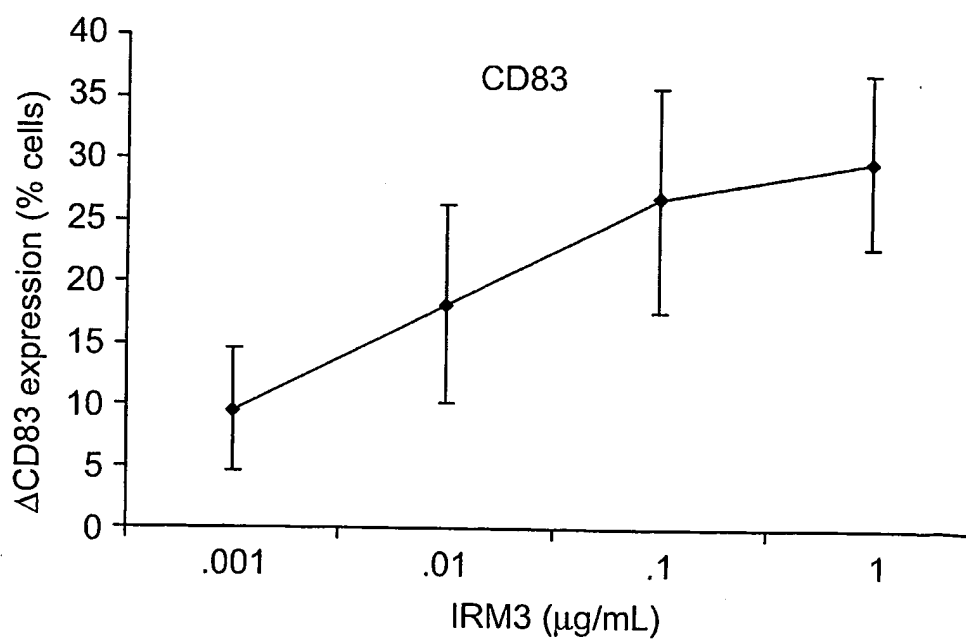
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*FIG. 5A**FIG. 5B*

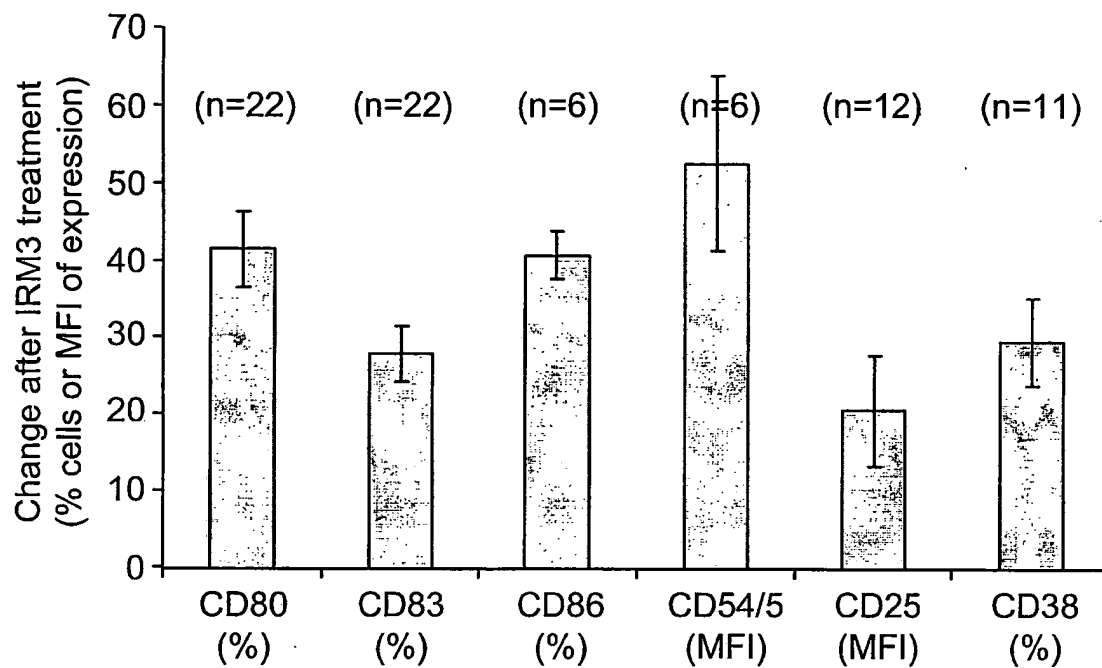
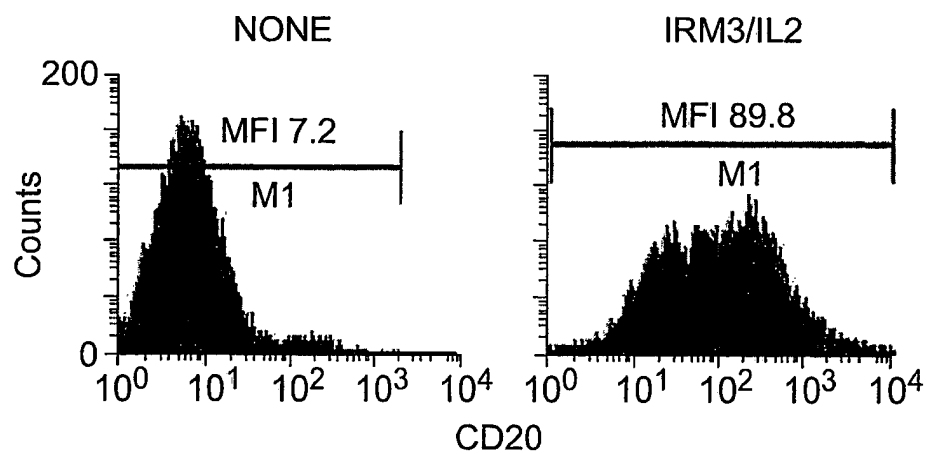
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*FIG. 6A**FIG. 6B**FIG. 7*

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*FIG. 8A**FIG. 8B*

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*FIG. 9**FIG. 10*